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MOLECULAR CHARACTERIZATION OF TI RINGSPOT ASSOCIATED EMARA VIRUS AND THE DEVELOPMENT OF ASSAYS FOR ITS DETECTION

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ABSTRACT

Ti ringspot disease (TRD) is an emerging virus-like disease of ti plants (*Cordyline fruticosa* L.) spreading throughout the Hawaiian Islands. Foliar symptoms include chlorotic lesions constricted by secondary veins or circular ring-spots that can coalesce into amorphous lesions. A degenerate reverse transcription (RT)-PCR assay targeting the polymerase gene of known emaraviruses amplified a product from the RNA of symptomatic but not asymptomatic ti plants. The RT-PCR products were sequenced and found to represent a new virus species, designated ti ringspot associated virus (TiRaV). Next generation sequencing of a double-stranded RNA library provided additional sequence information on TiRaV, allowing the development of detection assays able to reliably detect TiRaV and further associate it with TRD. Phylogenetic analyses of this sequence data revealed that TiRaV forms a clade with the emaraviruses *Raspberry leaf blotch virus* and *High Plains wheat mosaic virus* in the unassigned plant virus genus *Emaravirus*.

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CHAPTER 1

LITERATURE REVIEW

Botany of *Cordyline fruticosa*:

The ti plant, *Cordyline fruticosa* (L.), is a monocot with a secondary thickening in the stems and rhizomes (Simpson, 2000). The stem contains alternating fan-like leaves that grows around 0.5 m long. The blade is elongated and bright green and contains bisexual flowers on the terminal infloresences. Red round fruiting bodies are found in wild plants which were adapted freeboard dispersals and contain small black seeds. The stem of the plant is connected to rhizomes that grow vertically downwards and contain roots with fibrous side branches (Hinkle, 2007).

Classification of *C. fruticosa* has been frequently changed over the years. The genus was first collected and described by Rumpf in 1653 as *Terminalis* and was later changed to C*ordyline* by Robert Brown in Prodromes, 1810. In 1919, the name was officially changed to *Cordyline fruticosa* by Chevalier (Simpson et al 2013). The plant is still referenced by some as *Cordyline terminalis*, however The Plant List (2013) indicated that the name is invalid.

Ti plants are distributed around the world in tropical regions. The exact indigenous distribution is unclear due to the complicated human dispersal and selections. However, the ti plant is widely found in various continents and counties (Simpson, 2013). Introduction of *C. fruticosa* was thought to be from Papua or nearby by migrating Polynesians. Due to its simple propagations and easy dispersal through birds, the plant was spread widely. Sterile populations were created through selective breeding and were carried throughout eastern Polynesia (Hinkle, 2007).

Ethnobotany of Ti:

The ti plant is a common plant Polynesians have used for a variety of purposes for thousands of years. The Polynesians would utilize all parts of the plant. This includes roots for food additives and sweeteners, and leaves for roof thatching and clothing purposes such as skirts. Dancers would prefer ti-leaf skirts due to their rustling sounds when swished and a unique variety of movements. However, the leaves of the plant were also thought to possess supernatural qualities. Fire walkers in the South Pacific used ti-leaf skirts as a method of protecting themselves from the heat due to the sacredness towards the fire goddess. Furthermore, the leaves were used as a means of warding off ghosts and spirits by tying the leaf to objects (Craig, 2004., Ehrlich,1999). The ti leaf was also regarded as a religious symbol. For example, marriages would often be led by using ti leaves and sacred offerings would be wrapped in the leaf. Similar to its use by other Polynesians, the Hawaiian ti was used for basic commodities and religious practices. Ti was considered sacred to the Hawaiian god Lono and the Goddess of Hula Laka (Ehrlich, 1999).

Current propagation methods include stem cuttings and seeds. However, due to the sterility of the green ti plants found in Hawaii (Hinkle, 2007), stem cuttings are often the most common method for propagation of this variety. Stem cuttings are cut in to sections (4-6 inches) and place horizontally into soil until roots are established. The stem cuttings are then further cut (1 inch) and are placed into separate pots. Seed propagation includes gathering ripe seeds and sowing when fresh. This enables the seeds to rapidly germinate within 2-6 weeks. However unlike stem cutting, seed propagation creates a wide variation in plant size, leaf color, and shape (Kobayashi et al., 2007). However, new propagation methods have been reported. *In vitro*

propagation and *Agrobacterium*-mediated genetic transformation is present and may provide alternative methods of propagating and diversifying ti plants (Dewir et al., 2015).

Known Pests and Diseases:

Like most plants, *C. fruticosa* harbors a wide arrange of pests and diseases. Plants with pests or diseases often display symptoms which include foliar damages, wilting, deformities, leaf curling, chlorosis, stunting, and (in severe cases) death to the plant. Like many plants, aphids, mealybugs, scales, thrips, spider mites, whiteflies, and other insects have been known to infest ti plants (Kobayashi et al., 2007; Henley et al., 1991; Wong, 2007). For example, aphids utilize their stylets to ingest sap which cause speckles on the foliage, reduced color, stunting, wilting, and deformation of the leaves. However, in severe cases, leaf loss can occur. Unlike aphids, broad mites are difficult to see with the naked eye. The mites prefer young leaves and new growth and cause leaf curling, deforming, distortion, and bronzing. Most pests are a large concern due to their ability to cause damage (Table. 1) which may provide infection sites for pathogens.

Fungal and oomycete pathogens are common in ti plants. These pathogens create a variety of leaf spots and root rots. Fungi that cause these diseases include *Phyllosticta, Cercospora* and *Fusarium* species, while oomycetes include *Phytophthora* spp. Leaf spots (*Cercospora, Fusarium, Phyllosticta, Phytophthora*) usually occur during prolonged wet periods without wind. This can cause discoloration of the leaves and lead to leaf loss (Wong, 2007). Root rots (*Fusarium*) can cause more severe symptoms such as die-back to the cane. Once this occurs, the bark will come loose, roots will become fragile, and easily disintegrated. Severe cases can include the death of the plant (Henley et al., 1991; Moorman, 2015).

Table1. Known pests and symptoms of ti plants.

Pests	**Symptoms**	**Reference**
Aphids	Speckles on foliage, reduced color, stunting, wilting, deformed leaves. In severe cases, leaf loss occurs.	Kobayashi et al., 2007; Wong, 2007
Broad mites (*Polyphagotarso-nemus latus)* and two spotted mite(*Tetranychus urticae Koch*)	Curling, puckering, deformity, distortion, and bronzing. Wilting may occur.	Kobayashi et al., 2007; Henly et al., 1991; Wong, 2007
Carmine Spiders (*Tetranychus cinnabarinus*)	Graying and discoloration of leaves, Yellowing or speckling on foliage. In severe cases, loss of foliage, and plant death may occur.	Kobayashi et al., 2007
Fungus Gnats	Damages when larvae feeding on roots, root hairs, leaves (when in contact with soil)	Kobayashi et al., 2007; Henly et al., 1991
Chinese rose beetles (*Adoretus sinicus*)	Hole in leaves. Heavy infestations cause lace-like appearance of leaves	Kobayashi et al., 2007
Grasshoppers/ katydids	Consume foliage, leaving irregular appearences	Kobayashi et al., 2007
Mealybugs *Dysicoccus neobrevipes*	Stunting and death of plant parts. Honeydew and sooty mold are present.	Kobayashi et al., 2007; Henley et al., 1991; Wong, 2007
Nematodes	Poor growth, stunted, clorotic foliage, premature wiling, low vigor, thin canopy, leaf loss; swollen, knotted gnarling areas on the roots	Kobayashi et al., 2007; Wong, 2007
Scales *Pinnaspis buxi*	Yellowing or chlorotic streak that radiates from point of attachment	Kobayashi et al., 2007; Henley et al., 1991

Table 1. (Continued) Known pests and symptoms of ti plants.

Thrips Parthenolthrips dracaena, Chaetanaphothrips signipennis	Stippling of leaf tissues followed by gray leaf scars, bronzing, or silvering. Infested leaves become curled or distorted with calloused areas. When severe, russeting, graying, and stunting of leaves may occur.	Kobayashi et al., 2007; Henley et al., 1991; Wong, 2007
White flies	Small yellow spots where adults/ immature whiteflies have fed. In dens populations, leaves become yellowed and lower leaves are covered with black sooty mold.	Kobayashi et al., 2007; Henley et al., 1991
Pathogen: Fungi		
Leaf Spot (Cercospora, Phyllosticta, Fusarium)	Initially, rust-colored specks form. Many specks merge to form rectangular areas between leaf veins. Rectangular yellow to brown spots between veins on both surfaces of leaves. Affected leaves yellow and fall.	Kobayashi et al., 2007; Henley et al., 1991; Wong, 2007
Stem and Root Rot (Fusarium)	Causes die-back to the cane. Bark becomes loose, and lesions observed near the base of the stem. Internal bark tissue is generally dry. Roots are mushy and brown and easily disintegrate when handled. Soft, mushy rot at base of cuttings or rooted plants. Rotten area frequently has purplish or reddish margin.	Kobayashi et al., 2007; Henley et al., 1991 Moorman, 2015
Southern blight (Sclerotium rolfsii)	Fruiting bodies of the pathogen form all over the infected tissue and appear as small mustard seed-sized bodies. The white fan-like mycelium of the pathogen also forms over the plant.	Henley et al., 1991;
Pathogen: Oomycete		
Phytophthora leaf spot (Phytophthora parasitica)	Lesions form mainly on lower leaves. Water-soaked, brown, zonate areas with irregular margins.	Henley et al., 1991
Pathogen: Bacteria		
Erwinia blight (Erwinia chrysanthemi, E. carotovora pv. Carotovora)	Wet, mushy leaf spot and stem rot. Lesions on leaves and stems are usually water-soaked and slimy and eventually disintegration occurs	Henley et al., 1991

Table 1. (Continued) Known pests and symptoms of ti plants.

Bacterial Stripe (*Pseudomonas*)	Water-soaked, slender, long stripes between veins, becoming darker as disease progresses until tissue falls out	Kobayashi et al., 2007; Henley et al., 1991
Xanthomonas bacterial blight	Necrotic lesions with a yellow halo ring around the infected site	Wong, 2007
Pathogen: Viral		
Tomato spotted wilt virus (TSWV)	N/A	Cho et al., 1986
Cordyline Virus-1	No known symptoms	Melzer, et al 2011, 2013a, 2013b
Cordyline Virus-2	No known symptoms	Melzer, et al 2011, 2013a, 2013b
Cordyline Virus-3	No known symptoms	Melzer, et al 2011, 2013a, 2013b
Cordyline Virus-4	No known symptoms	Melzer, et al 2011, 2013a, 2013b

Similar to most plants, bacterial pathogens are also a concern to ti growth and production. These pathogens include *Pseudomonas, Erwinia,* and *Xanthomonas* species. *Pseudomonas* spp. are the main cause of bacterial stripe which occurs during wet environments with little wind. This causes long stripes along the leaf veins which become darker as the disease progresses (Kobayashi et al., 2007). *Erwinia* spp. are the causal agent of bacterial stem rot which also occurs during wet environments. Stem rot causes the leaves and stem to be wet and slimy while roots of established plants become black and die (Moorman, 2015; Henley et al., 1991). *Xanthomonas* spp. are the causal agent of leaf blight (Wong, 2007). Symptoms of this disease would include necrotic lesions with yellow halo rings around the infected site. Both fungal and bacterial pathogens can affect plant health and morphology.

A total of five viral pathogens infecting ti have been reported, all of which were found in Hawaii. These five pathogens include *Tomato spotted wilt virus* (TSWV) and a recently found assemblage of related viruses termed Cordyline virus 1 (CoV-1), CoV-2, CoV-3, and CoV-4. TSWV has been detected on ti plants through ELISA. Three of the six plants tested with the bioassay gave positive readings, indicating that TSWV was present in the plant (Cho et al., 1986). The CoVs were later found in 2011 through molecular analyses (Melzer, et al 2011, 2013a, 2013b).

Ti Ringspot:

Severe symptoms can lead to complete yellowing of the leaf and eventually leaf death. Ti ringspot was first observed by growers in windward Oahu in 2009. Symptoms of this emerging disease include chlorotic lesions encompassed by secondary veins or circular ring-spots that coalesce into amorphous lesions (Figure l). To search for the causal agent of this disease,

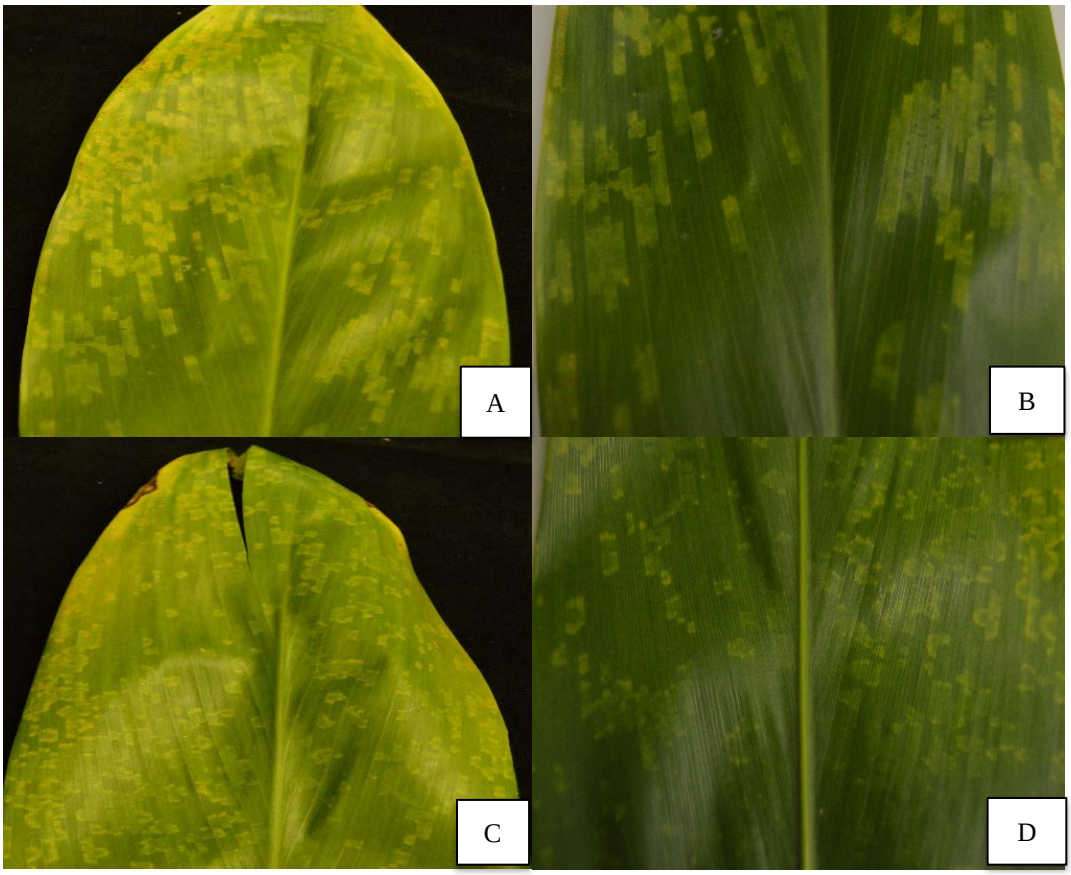

Figure 1. Different visual symptoms of Ti leaves. Symptoms may have (A/B) chlorotic lesions by secondary veins or circular ring-spots that coalesce into amorphous lesions (C/D). *B and D provide close up visuals of the symptoms

molecular analysis was done that included double stranded (ds)RNA analysis with massively-parallel sequencing. These efforts resulted in the discovery of CoV-1 to -4 (Melzer et al., 2011, 2013a, 2013b), however, it was determined that these viruses were not involved with the etiology of the ringspots (Melzer et al., 2010, 2013b). Transmission electron microscopy (TEM) of symptomatic tissue showed a double membrane-bound bodies similar to those of members of the genus *Emaravirus* (Figure 2; Melzer unpublished). Viruses in these genus can produce ringspot symptoms in their host, similar to those of ti ringspot (Mielke-Ehret et al., 2012).

Although still used for cultural and religious practices, today in Hawaii most ti leaves are used in the food industry as a traditional food wrap. Starting in 2009, ti ringspot symptoms on ti leaves reduced their value for the food industry and were no longer acceptable for ornamental purposes. Although the ti leaf industry has only a small commercial value of $541,000 from 2004-2008 (Hudson et al., 2008), the ringspots are causing many of the leaves to be unsellable, hurting local growers.

Emaravirus

Emaravirus is an unassigned genus of plant viruses (Muhlbach and Mielke-Ehret ,2011). The genus was established in 2011 and its members have a double membrane bodies (DMBs) between 80 and 200 nm in diameter that are observed near the endoplasmic reticulum and golgi cisterns (Silvestro et al 2004; Kumar et al., 2002). Members of the genus possess multiple single stranded, negative sense genomic RNAs. The genome is broken down into seven to eight negative sense genomic RNA segments, each encoding one ORF in the complimentary sense. RNAs 1, 2, 3, and 4 encode by RdRp, glycoprotein precursors, nucleocapsid, and movement proteins respectively (Mielke-Ehret and Mülbach, 2012).

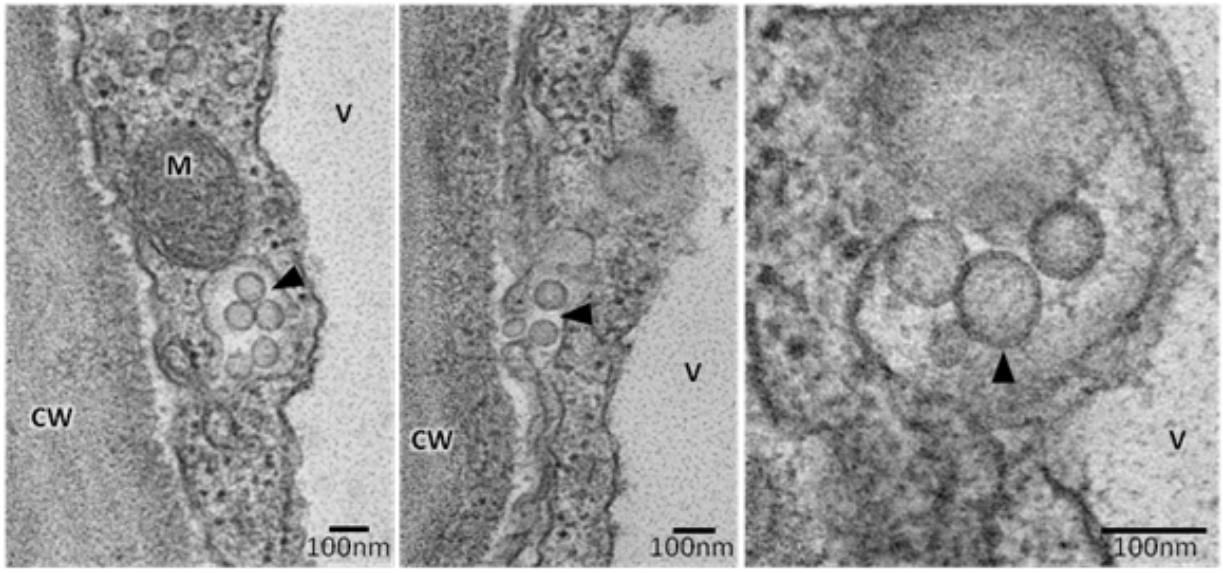

Figure 2. Transmission electron micrographs of double-membrane bodies. Bar represents 100 nm (Melzer et al., unpublished)

Table 2: Structural and biological characteristics of emaravirus-related viruses (Mielke-Ehret et al., 2012)

Virus Species	Particle Morphology	Vector (Putative)	Host Species
EMARaV	DMBs 80-120 nm	(*Phytoptus pyri*)	*Sorbus aucuparia*
FMV	DMBs 90-200 nm	*Aceria ficus*	*Ficus carica*
RRV	DMBs 120-150 nm	*Phyllocoptes fuctiphilus*	*Rosa multiflora* Cultivated hybrid roses
RLBV	Indistinct filamentous bodies	*Aceria tosichella*	*Rubus spp.*
PPSMV	Filamentous structures and DMBs 100-150 nm	*Aceria cajani*	*Cajanus cajan*
HPWMoV	DMBs 100-200 nm	*Aceria tosichella*	*Triticum aestivum*

The genus *Emaravirus*, is currently composed of six known species: *European mountain ash ringspot-associated virus* (EMARaV) affecting European mountain ash (*Sourbus aucuparia*), *Fig mosaic virus* (FMV) affecting figs (*Ficus carica*), *Rose rosette virus* (RRV) affecting cultivated hybrid roses (*Rosa multiflora*), *High Plains Wheat mosaic virus* (WMoV) affecting wheat (*Triticum aestivum* L.), *Raspberry leaf blotch virus* (RLBV) affecting raspberry and other *Rubus spp.*, and *Pigeonpea sterility mosaic virus* (PPSMV) affecting pigeon pea (*Cajanus cajan*) (Mielke-Ehret and Mülbach., 2012; Laney et al., 2011; Serrano et al., 2004; Tatineni et al., 2014; Mcgavin et al., 2012; Kumar et al., 2005). Further characteristics of the virus genus are summarized in Table 2. Current putative emaraviruses include: Redbud yellow ringspot-associated virus (RYRV) (Laney et al., 2010), Woolly burdock yellow vein virus (WBYVV) (Bi et al 2012), and Pigeonpea sterility mosaic virus 2(PPSMV-2) (Elbeaino et al., 2015). Sequence comparison analysis of current members of *Emaravirus* (EMARaV, FMV, RLBV, RRV, and PPSMV) showed high percentage amino acid (aa) and nucleotide identities for the RdRp which ranges from 32.9% to 48.3% and 48.5% to 55.4% respectively. FMV seems to have the highest amino acid identity with EMARaV at 48.3% followed by RRV and PPSMV at 48.2% and 47.3% compared to RLBV with only 32.9%. Due to these identity similarities, clades are formed with EMARaV, FMB, PPSMV, and RRV while RLBV is typically within a clade formed with HPWMoV.

Diseases caused by RRV, RLBV, FMV, and PPSMV are all known to be transmitted by eriophyid mites. These mites are a group of arthropods that are host specific and serve as vectors for several potyviruses (Figure 3) (Amrine et al., 1998; Jones et al., 1984; Flock et al 1955; Kulkarni et al 2002). Although most members of the genus are transmitted by eriophyid mites, vector transmission by an eriophyid mite has not been shown for EMARaV. Instead, quantitative

RT-PCR and immune fluorescence microscopy has shown that both genomic and complementary forms of RNA 3 (P3: nucleocapsid) were present inside the pear leaf blister mite (*Phytoptus pyri*), providing vector candidacy for EMARaV (Mielke-Ehret et al., 2010). Eriophyid mites, living on the undersurface of the plant leaves, contain short stylets that reach epidermal cells or the adjacent layers of the mesophyll. For effectiveness of the virus transmission, the cells types described must be infected (Mielke-Ehret et al., 2012).

Figure 3. Eriophyid mite collected from ti.

Table 3. Sequence identity matrix of all genomic RNA segments of all emaraviruses. Current (in bold) and putative members are determined from nucleotide and deduced amino acid (in bold) sequences of multiple RNA segments of emaraviruses. (Elbeaino et al., 2015.)

Emaravirus	RNA segment	Accession number	**EMARaV**	**FMV**	**RRV**	**PPSMV**	**RLBV**	HPWMoV	RYRV	PPSMV-2
EMARaV	1	AY563040		54.7	55.4	54.9	48.5	45.5	59.6	55.5
	2	AY563041		48.5	47.9	46.8	38.8	35.3	48.9	48.1
	3	DQ831831		44.2	42.2	41.2	33.6	34.1	49.0	37.9
	4	DQ831828		33.8	32.8	34.1	28.6	29.2	35.7	33.4
FMV	1	AM941711	**48.2**		67.7	59.0	48.4	45.8	55.2	70.5
	2	FM864225	**35.8**		59.7	53.5	39.6	34.5	50.6	62.2
	3	FM991954	**36.7**		57.5	46.7	34.8	34.7	42.0	56.7
	4	FM992851	**6.0**		60.7	46.3	35.2	32.9	44.7	68.7
RRV	1	NC_015298	**48.3**	**67.9**		67.7	48.4	45.7	54.6	67.6
	2	NC_015299	**35.7**	**49.0**		59.7	38.8	35.7	51.3	59.8
	3	NC_015300	**31.1**	**59.5**		57.5	35.1	36.7	41.6	50.7
	4	NC_015301	**6.6**	**59.0**		60.7	38.4	35.0	45.6	60.8
PPSMV	1	HF568801	**47.3**	**51.5**	**52.9**		48.0	45.2	54.9	62.5
	2	HF568802	**37.0**	**43.3**	**42.3**		41.5	35.9	50.0	51.1
	3	HF568803	**32.4**	**39.6**	**39.9**		38.1	36.6	43.9	48.5
	4	HF568804	**5.2**	**38.2**	**38.2**		37.9	35.6	42.6	49.4
RLBV	1	FR823299	**32.9**	**31.8**	**31.9**	**32.0**		55.8	48.4	48.7
	2	FR823300	**22.0**	**22.9**	**22.3**	**25.2**		51.6	38.9	39.5
	3	FR823301	**20.2**	**20.3**	**19.3**	**23.0**		48.4	36.0	34.0
	4	FR823302	**5.8**	**16.7**	**19.8**	**19.6**		54.8	36.7	37.3
HPWMoV	1	KJ939623	**30.8**	**30.6**	**31.0**	**30.1**	**41.8**		45.3	46.0
	2	FR823300	**20.1**	**20.8**	**22.1**	**21.7**	**33.9**		35.6	35.0
	3	KJ939625	**15.1**	**17.9**	**16.1**	**18.4**	**26.6**		35.6	34.0
	4	KJ939627	**5.4**	**17.7**	**19.5**	**18.5**	**44.0**		33.4	34.4

Table 3. (Continued) Sequence identity matrix of all genomic RNA segments of all emaraviruses. Current (in bold) and putative members are determined from nucleotide and deduced amino acid (in bold) sequences of multiple RNA segments of emaraviruses. (Elbeaino et al., 2015.)

RYRV	1	JF795479	**53.8**	**46.7**	**46.4**	**46.8**	**33.2**	**31.4**		54.3
	2	JF795480	**41.4**	**38.1**	**38.5**	**39.4**	**21.8**	**22.0**		50.5
	3	JF795481	**44.6**	**35.8**	**33.1**	**33.2**	**18.2**	**17.8**		42.2
	4	JF795482	**5.2**	**28.7**	**30.3**	**25.9**	**19.6**	**14.8**		45.0
PPSMV-2	1	HF912243	**48.9**	**72.1**	**67.5**	**52.9**	**32.8**	**30.9**	**46.4**	
	2	HF912244	**34.0**	**57.1**	**51.7**	**42.7**	**21.0**	**20.3**	**38.0**	
	3	HF912245	**35.6**	**80.0**	**60.6**	**39.8**	**18.8**	**16.0**	**33.2**	
	4	HF912246	**6.0**	**75.0**	**61.2**	**39.3**	**18.0**	**17.9**	**30.8**	

CHAPTER 2

ETIOLOGY OF TI RINGSPOT DISEASE AND MOLECULAR CHARACTERIZATION OF TI RINGSPOT-ASSOCIATED EMARA VIRUS

Ti ringspot disease (TRD) is an emerging disease affecting ti plants (*Cordyline fruticosa*) in Hawaii. First observed by growers in 2009, symptoms occur primarily on older leaves and include chlorotic lesions or circular ringspots that can coalesce into amorphous lesions (Chapter 1, Figure 1). Several velariviruses have recently been discovered in symptomatic ti plants (Melzer et al 2011, 2013a, 2013b), however they were also present in asymptomatic plants, making them unlikely to be involved in the etiology of TRD (Melzer et al., 2013). Transmission electron microscopy (TEM) of symptomatic tissues containing revealed the presence of double membrane-bound bodies similar to those of members of the genus *Emaravirus* (Park et al., unpublished). These structures were not observed in asymptomatic tissues.

The *Emaravirus* is an unassigned genus of plant viruses (Muhlbach and Mielke-Ehret, 2011). The genus was established in 2011 and its members have a double membrane bodies (DMBs) between 80 and 200 nm in diameter that are observed near the endoplasmic reticulum and golgi cisternae (Silvestro et al 2004; Kumar et al., 2002). Members of the genus possess a single stranded, negative sense genomic RNA. The genome is broken down into seven to eight negative sense genomic RNA segments, each encoding one ORF in the complimentary sense. RNAs 1, 2, 3, and 4 encode by RdRp, glycoprotein precursors, nucleocapsid, and movement proteins respectively (Mielke-Ehret and Mülbach, 2012).

The genus *Emaravirus*, is currently composed of five known species: *European mountain ash ringspot-associated virus* (EMARaV), *Fig mosaic virus* (FMV) affecting figs (*Ficus carica*),

Rose rosette virus (RRV), *Raspberry leaf blotch virus* (RLBV), and *Pigeonpea sterility mosaic virus* (PPSMV) (Mielke-Ehret and Mülbach., 2012; Laney et al., 2011; Serrano et al., 2004; Mcgavin et al., 2012; Kumar et al., 2005). Current putative *Emaraviruses* include: Redbud yellow ringspot-associated virus (RYRV) (Laney et al., 2010), High Plains wheat mosaic virus (HPWMoV) (Tatineni et al., 2014), Woolly burdock yellow vein virus (WBYVV) (Bi et al 2012), and Pigeonpea sterility mosaic virus 2(PPSMV-2) (Elbeaino et al., 2015).

The specific objectives of this chapter are to i) determine whether TRD is associated with emaravirus infection ii) determine the genetic diversity of emaraviruses infecting ti, and iii) determine if any emaraviruses infecting ti are existing or previously undescribed species.

Materials and Methods

Sample Collection. Leaves from fourteen symptomatic plants were collected from seven different locations (Figure 4) across Hawaii (Oahu, Maui, and Hawai'i) along with five asymptomatic plants. These leaf samples were labeled, cataloged, and stored at 4 ^{o}C until processed.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from leaf tissues using a NucleoSpin®RNA II Kit (Macherey-Nagel, city, state) following the manufacturers protocol with the following modification. Tissue volume was increased from 30mg to 100mg, buffer RA1 and β-mercaptoethanol were increased from 350 µL to 900 µL and 3.5 µL to 9 µL respectively, and cells were lysed using a Mini BeadBeater 16 (Biospec, Bartlesville, OK). Extracted RNA was then used as template for cDNA synthesis.

RT-PCR. To synthesize cDNA template for PCR, 1µL (10 pmol) of random hexamer primer followed by 6.5 µL of nuclease free H_2O and 2 µL of RNA were denatured at 72^{o}C for

Figure 4. Map of sampling locations.

10 minutes and then quickly chilled on ice. After the addition of 5 µL of dNTPS (2mM each), 4 µL of 5x MMLV buffer (Promega, Madison, WI), 1 µL MMLV Reverse Transcriptase (Promega), and 0.5 uL of RNasin® (Promega), the mixture was incubated at 25 °C for 10 min then 42 °C for 50 min.

PCR was performed using degenerate primers (motif A sense and motif C antisense) that target conserved regions in the RNA-dependent RNA polymerase (RdRp) gene of emaraviruses (Elbeaino et al., 2012). PCR reactions were created using 10 µL of GoTaq Green Master Mix (Promega), 1 µL of cDNA template, 1 µL of forward (10 pmol) and 1 µL of reverse (10 pmol) emaravirus-specific degenerate primers, and 7 µL of H_2O. Amplification was performed using a Veriti® 96 well thermocycler (Applied Biosystems; Foster City, CA) with the following conditions: one cycle of 94 °C for 5 min; forty cycles of 94 °C for 30 secs, 48 °C for 30 secs, 72 °C for 40 secs; and one cycle of 72 °C for 7 mins (Figure 5). PCR products were then resolved on a 1% agarose gel containing ethidium bromide and viewed using UV light.

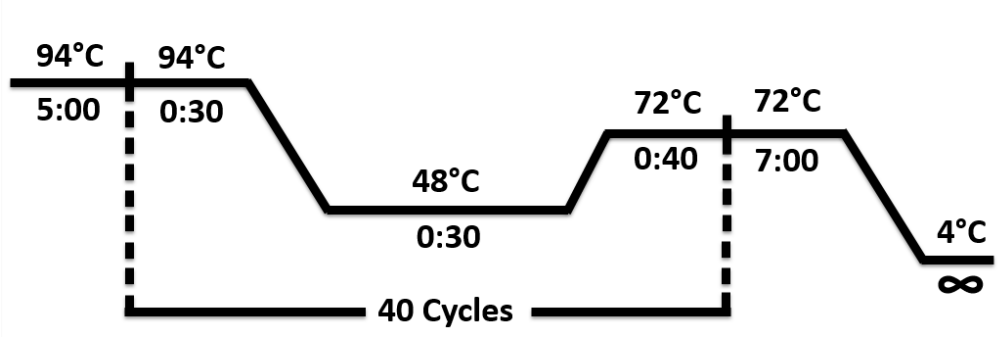

Figure 5. RT-PCR conditions for emara-specific degenerate primers.

Cloning and Sequencing. RT-PCR products of the expected size were excised with a razor blade and placed in a 0.6 mL centrifuge tube with a pinhole in the bottom covered by a small piece of GF/C filter paper (Whatman, Buckinghamshire, UK). The tube was placed in a 1.5

mL centrifuge tube and centrifuged at 12,000 *g* for 2 mins. The eluate containing the purified cDNA was used directly for cloning vector ligation. The gel-purified amplicons were cloned using a pGEM-T Easy system (Promega; Madison, WI) following the manufacturer's protocol. The ligation reaction was incubated either at 14^{o}C overnight or at room temperature (~23 ^{o}C) for 2 h. *E. coli* DH5α cells were transformed with 5 µL of the ligation reaction and placed on ice for 60 mins. The reaction was placed in a 42^{o}C water bath for 45 s, and placed back on ice. Following the addition of 400 µL of SOB the tube was incubated at 37^{o}C for 45 min. The cells were plated on MacConkey agar (Sigma) containing 75 to 150 ppm of ampicillin and incubated for 24 hours at 38 ^{o}C. At least three colonies containing recombinant plasmids from each plate were selected and underwent plasmid mini-preps using a QIAprep Spin Miniprep kit (Qiagen; Valencia, CA) following the manufacturer's instructions.

Sequence Analysis. Plasmid DNA inserts were sequenced from both directions using SP6 and T7 primers. Automated sequencing was conducted at the Advanced Studies in Genomics, Proteomics and Bioinformatics, University of Hawaii, Honolulu, HI.

Sequence data was analyzed using Chromas lite (Technelysium). BLASTX was used to translate nucleotide to amino acid sequences and compare the products with the amino acid sequence database at the National Center for Biotechnology Information (NCBI) website. Sequences from the same tissue sample were assembled into a consensus sequence using CAP3 (Huang and Madan, 1999). These consensus sequences, each representing a sample gathered from a different location were compared using LALIGN (Huang and Miller, 1991) and multiple sequence alignment was performed with Clustal X2 (Larkin et al. 2007).

Phylogenetic Analysis. Phylogenetic analyses were done using SeaView, Version 4 (Gouy et al. 2010) with all members of the genus *Emaravirus* with *Tomato spotted wilt virus* (TSWV; Family *Bunyaviridae*, Genus *Tospovirus*) as an outgroup. A total of three different phylogenetic algorithms were used to determine consistency: Maximum-likelihood and neighbor joining. All algorithms were bootstrapped with 1000 replications. Amino acid sequences of other emaraviruses and outgroups were obtained through NCBI (Table 4).

Results

RT-PCR. Fourteen symptomatic plants from seven locations across Hawaii along with five asymptomatic ti plants were screened for emaravirus infection using a degenerate RT-PCR assay. RT-PCR products of the correct size (~400 bp) were generated from all symptomatic plants while five asymptomatic and non-template control reactions did not produce amplification products (Figure 6).

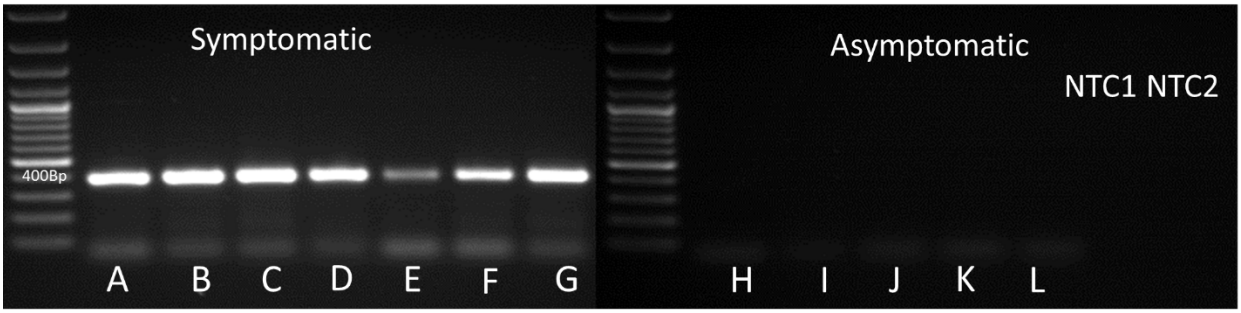

Figure 6. Amplification products (400bp) from leaf samples taken from various locations across Hawaii. Symptomatic locations include Imiloa (A), North Kohala (B), Richardson Beach Park (C), Manoa (D), Waikapu (E),Kapaniwai (F), Waimanalo (G). Four asymptomatic samples (H, I, J, K, and L) were obtained from clean tissue cultured ti plants. NTC is a non-template (water) control.

Table 4. Abbreviation and genbank accession numbers for all emaraviruses.

Virus	Abbreviation	Genbank accession number
Ti Virus	N/A	XXXXXXX
Raspberry leaf blotch virus	RLBV	FR823299
High Plains wheat mosaic virus	HPWMoV	KJ939623
Rose rosette virus	RRV	NC_015298
Fig mosaic virus	FMV	AM941711
European mountain ash ringspot-associated virus	EMARaV	AY563040
Pigeonpea sterility mosaic virus	PPSMV	HF568801
Pigeonpea sterility mosaic virus-2	PPSMV-2	HF912243
Redbud yellow ringspot-associated virus	RYRV	JF795479

Genetic Diversity and Phylogenetic Analyses. Select RT-PCR products were cloned and sequenced, with at least three clones used to produce a consensus sequence. The consensus sequence from each of samples collected from seven different locations across the state of Hawaii was obtained (Figure 4). LALIGN results showed these seven consensus sequences were >98% identical (Figure 7).

Consensus sequences were compared to the non-redundant database in GenBank using the BLASTX algorithm. Putative conserved domains typical of bunyavirus RNA-dependent RNA polymerases (RdRp) were detected in the sequence, which was found to be most closely related to members of the genus *Emaravirus*, an unassigned viral genus (ICTV; Mühlbach and Mielke-Ehret). The sequence was most closely related to RLBV with a 67.8% amino acid sequence identity over the region.

Sequence of the virus in ti and known and tentative emaraviruses were analyzed to determine their phylogenetic relationships. Both maximum-likelihood (ML) and neighbor-joining (NJ) algorithms indicated the ti virus formed a distinct branch in a clade with RLBV and HPWMoV. The other emaraviruses and tentative emaraviruses formed a second clade (Figure 8).

Discussion

TRD is a new disease with little information on its etiology available. Melzer et al. (2013) determined the symptoms were not caused by velarivirus infection, therefore supporting the presence of another agent responsible for the disease. Symptoms of TRD are similar to the ringspot associated with the ringspot disease of European mountain ash and fig mosaic disease caused by EMARaV and FMV, respectively. In addition, the presence of emaravirus-like DMBs in symptomatic tissues (Park et al. unplublished) suggest the agent responsible for TRD

may be an emaravirus. In this study, symptomatic and asymptomatic isolates gathered from different parts of the state were tested using emaravirus-specific degenerate primers. The degenerative primers created by Elbeaino et al. (2012) were selected from highly conserved regions of the RdRp (RNA 1) gene found in all five emaraviruses (FMV, EMARaV, RLBV, RRV, RYRV). These highly conserved RdRp regions, denoted as pre-motif A, motif A, and motif C, were used to design primer sets that target viruses in the *Emaravirus* genus. Although not all primer sets designed by Elbeaino et al. (2012) were utilized, motif A-sense and motif C-antisense produced visible bands of the expected ~400bp size for symptomatic isolates, indicating that the virus in Hawaiian ti plants has similar conserved regions as other emaraviruses. Asymptomatic ti plants produced no visible bands and suggest the agent detected by the degenerate emaravirus primers is associated with TRD.

Sequencing of the RT-PCR products followed by BLASTX analysis showed putative conserved domains of the viral RdRp region that is targeted by the emaravirus degenerative primers. The RT-PCR product from ti had 67.8% amino acid identity to, suggesting that RLBV is the closest relative to the ti virus. Supporting this observation were the results of the phylogenetic analysis where RLBV, HPWMoV, and the virus in ti share a clade. Phylogenetic work done by Tatineni et al. (2014) on HPWMoV produced similar results in that RLBV and HPWMoV form a distinct clade within the genus *Emaravirus*.

The RT-PCR products from ti plants with TRD from several locations across the state were sequenced to determine the genetic diversity of this putative virus. All products had high sequence homology (98-99%) with each other, implying only a single agent is responsible for TRD in Hawaii, and there is little genetic diversity in the sequenced region for this viral agent.

The proposed species demarcation criteria for members of the genus *Emaravirus* are i) differences in relevant gene product sequences of more than 25%, ii) differences in host ranges, and iii) differences in vector specificities (Tatineni et al, 2015). With these criteria in mind, the RT-PCR product sequence, when converted to amino acid sequence, was more than 25% divergent from tentative and putative members of the *Emaravirus* genus. Additionally, none of the described emaravirus species are known to infect ti, suggesting this virus has a distinct host range from existing emaravirus species. At present, there is no information available on any vector associated with TRD. Based on these criteria and the phylogenetic analyses conducted in this chapter, as well as previous observations of DMBs in infected tissue, it is concluded that the viral agent associated with TRD represents a new species of the genus *Emaravirus*. The tentative proposed name of this virus is Ti ringspot associated virus (TiRaV).

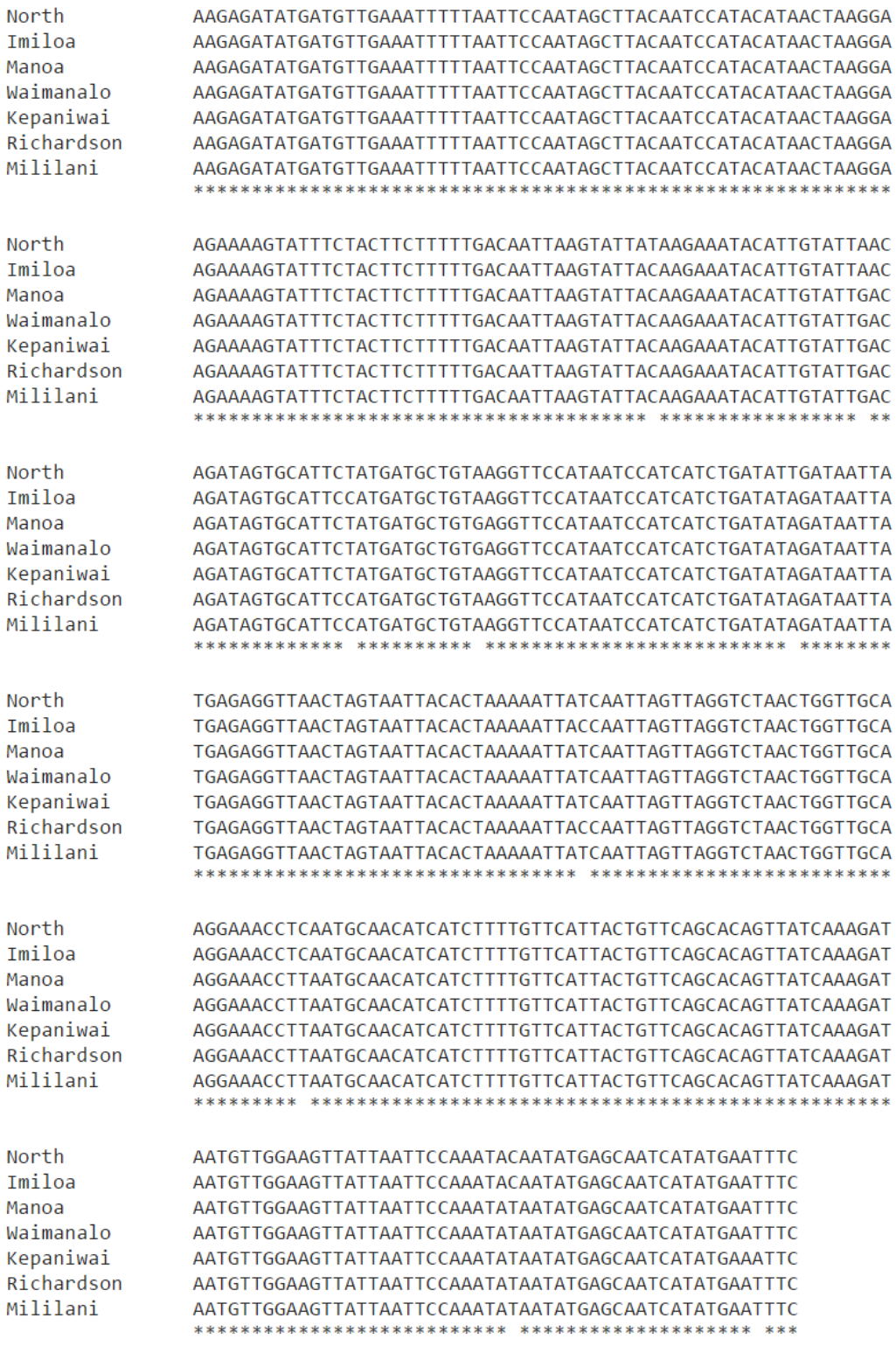

Figure 7. ClustalW alignment of RT-PCR product sequences from different locations using degenerate emaravirus detection primers.

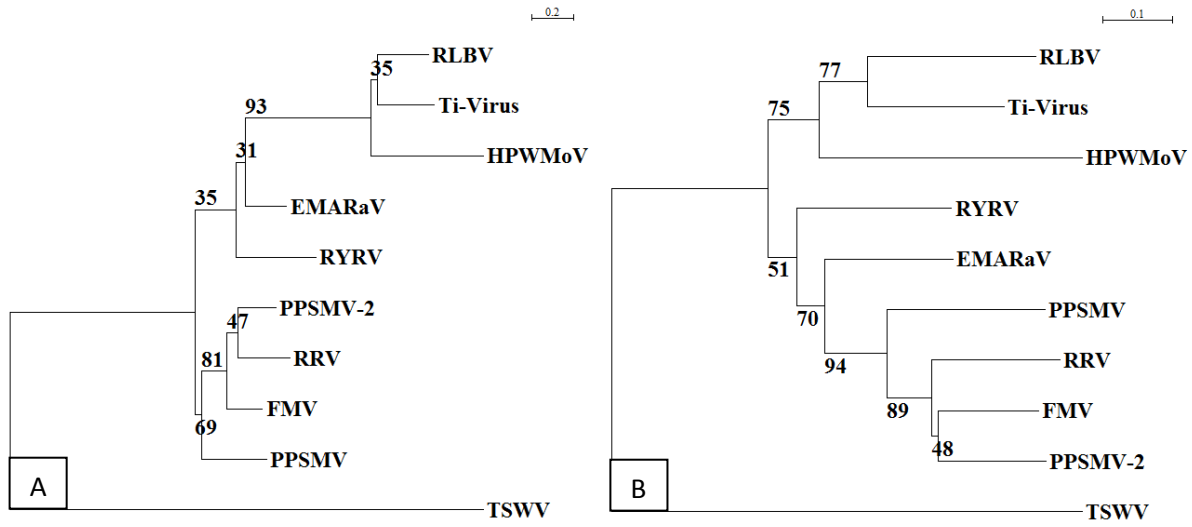

Figure 8. Phylogenetic analysis of Ti Virus with maximum-likelyhood (A) and neighbor-joining (B). A 1000 bootstrap value was used for both methods.

CHAPTER 3

DOUBLE STRANDED RNA ISOLATION AND PARTIAL SEQUENCNG OF THE TI RINGSPOT ASSOCIATED VIRUS GENOME

Ti ringspot associated virus (TiRaV) is a new and understudied virus. Although RT-PCR, sequence homology, and phylogenetic analysis indicate TiRaV is most closely related to members of the unclassified virus genus *Emaravirus*, most of the genomic sequence of the virus is unknown. In order to develop a detection assays and control strategies, more of the genomic sequence must be obtained. As described in CHAPTER 2, a short sequence of the TiRaV RdRp gene was amplified using degenerate primers developed by Elbeaino et al. (2012). These degenerate primers produced ~400 bp product from a single gene, making conclusions on the taxonomic placement of TiRaV difficult. Furthermore, this short sequence may be insufficient to develop robust molecular-based detection assays for the virus. In this chapter, a library derived from double-stranded (ds)RNAs isolated from ti plants infected with TiRaV are used in next generation sequencing (NGS) to increase the amount of sequence data available for the TiRaV genome.

Materials and Methods

Isolation and Analysis of TiRaV dsRNA. Double-stranded RNA was isolated from TiRaV-infected ti plants using a modified protocol dsRNA protocol (Morris and Dodds. 1979, Bar-Joseph et al. 1983, and Dodds et al. 1984). Approximately 5 g of leaf and stem tissues of TiRaV-infected plants was frozen in liquid nitrogen and then finely grounded using a pre-cooled mortar and pestle. The powered tissue was added to 200 mL of dsRNA extraction buffer (18 mL 10X STE, 30 mL 10% SDS, 2mL β-mercaptoethanol, 60 mg Bentonite, 70 mL dH_2O, 40 mL saturated phenol, and 40 mL chloroform) and was stirred for 45-60 min at 4°C with a magnetic

stir bar. The contents were then transferred to a 250 mL GSA bottle and centrifuged 8000 rpm for 10 min. The aqueous layer was transferred to a new bottle and the volume was recorded. 0.2 volumes of EtOH and 1 g of CF-11 cellulose (Whatman; Maidstone, England) was added and gently shaken over night at room temperature. The sample was then passed through a vertically clamped 60cc syringe barrel containing a miracloth filter (Calbiochem; La Jolla, CA). 100 mL of 1x STE containing 16.5% of EtOH and was added to the syringe and was purged using the syringe plunger. The cellulose was then eluted with five 5 mL aliquots of 1x STE and purged after each elution. 0.2 volumes of EtOH and 1g of cellulose was added to the sample and left gently shaken for an hour. The sample was then transferred to another column containing a miracloth filter. 100 mL of 1x STE containing 16.5 % EtOH was added to the syringe and was purged. Three 3 mL aliquots of 1x STE was added and eluted into a 15 mL centrifuge tube. Any contaminating cellulose was pelleted by centrifugation, and the supernatant was transferred to a 30 mL cortex centrifuge tube where it was precipitated by adding 0.9 mL of 3M NaAc (pH 5.2), and 2 volumes of 95% EtOH, and stored overnight at -20°C. Samples were centrifuged at 12000 rpm for 30 min, the supernatant discarded, and the pellet washed with 500 µL of RNase-free H_2O. The dsRNA was concentrated using a Amicon Ultra filter (Millipore, Darmstadt, Germany).

dsRNA Library. To synthesize the cDNA template, 2 µL of dsRNA and 1 uL of random primer (5'- GCC GGA GCT CTG CAG AAT TCN NNN NN-3') was denatured at 95 °C for 8-10 min, and quickly chilled on ice. After the addition of 4 µL AMV RT Buffer (Promega, Madison, Wisconsin), 1 µL of AMV enzyme (Promega, Madison, Wisconsin), 4 µL dNTPS (2mM each), and 8 µL of H_2O was added. The reaction was incubated to 37 °C for 90 min. Overlap extension PCR was performed by adding 20 µL of GoTaq to the cDNA reaction. Amplification was

performed using a Veriti 96 Well Thermocycler (PE Applied Biosystems; Foster City, CA) and involved: one cycle of 95 °C for 7 mins, ten cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min; and 72 °C for 7 min. To obtain the dsRNA library, an anchor primer PCR was performed with 10 µL of GoTaq, 7 µL of H_2O, 2 µL of universal primer (5'- GCC GGA GCT CTG CAG AAT TC-3') and 1 µL of the overlap extension PCR. Amplification was preformed using the same thermocycler but involved: one cycle of 95 °C for 7 mins, thirty-five cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min; and 72 °C for 7 min. Any PCR products synthesized were resolved on a 1% agarose gel, and represented the dsRNA library (Figure 9).

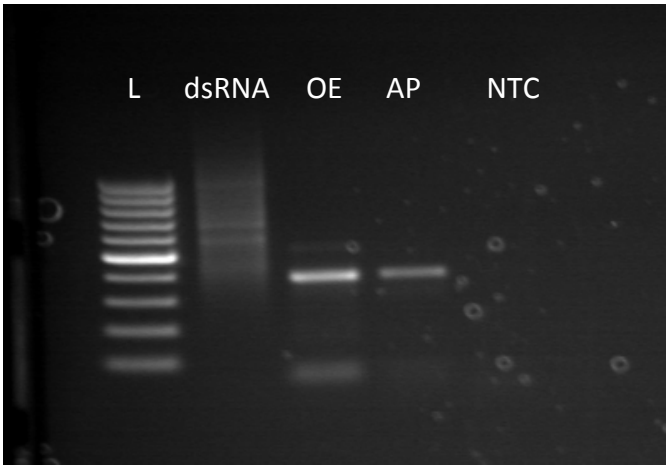

Figure 9. DsRNA library resolved on 1% agarose gel. Overlapping extension (OE) PCR and anchor primer (AP) PCR products utilizing degenerative primers are also shown to represent the presence of TiRaV within the library. NTC is a non-template (water) control.

Next Generation Sequencing and Analysis. The dsRNA library was concentrated using a YM-30 or YM-50 Amicon Ultra centrifugal filter unit following the manufacturer's instructions. Concentrated products were submitted for next generation sequencing (NGS) at SeqMatic (San Francisco, California). The library was sequenced on an Illumina MiSeq V3 sequencing platform

with paired end read sequencing (2x300 bp). Sequence data were analyzed using two different methods: Virfind (Ho and Tzanetakis. 2014) and Geneious 6.1.8 (Biomatters Limited, Auckland, New Zealand). Virfind results were automatically given with a BLAST (Altschul et al., 1990, 1994) output. Sequences placed into Geneious were manually analyzed. Raw sequences underwent *de novo* assembly into contiguous sequences (contigs) and non-TiRaV sequences were removed through assembly to existing reference genomes or through BLASTX analysis. Contigs with putative emaravirus domain hits were then mapped to the emaravirus *Raspberry leaf blotch virus* (RLBV). The amino acid sequence homology of TiRaV contigs was determined for all known members of the genus *Emaravirus* using LALIGN (Huang and Miller, 1991).

Phylogenetic analyses. Phylogenetic analyses were performed on TiRaV amino acid residues linked together into a single chimeric sequence using SeaView, Version 4 (Gouy et al. 2010). Included in the analyses were the corresponding sequences from all members of the genus *Emaravirus* and *Tomato spotted wilt virus* (TSWV; Family *Bunyaviridae*, Genus *Tospovirus*) as an outgroup. Two different phylogenetic algorithms were used to determine consistency: maximum-likelihood and neighbor joining (SeaView, Version 4). All algorithms were bootstrapped with 1000 replications. The amino acid sequences of other emaraviruses and the TSWV outgroup were obtained from GenBank.

Results

Next Generation Sequencing and Analysis. NGS of the dsRNA library resulted in a total of 981,006 single reads at 301 nt. Single reads were placed into VirFind pipeline through BLASTX where a total of 6 contigs (V1-46452, V1-53644, V1-53643, V2-74357, V2-74359, V2-74358) were a match with known emaraviruses. All contigs derived from VirFind were

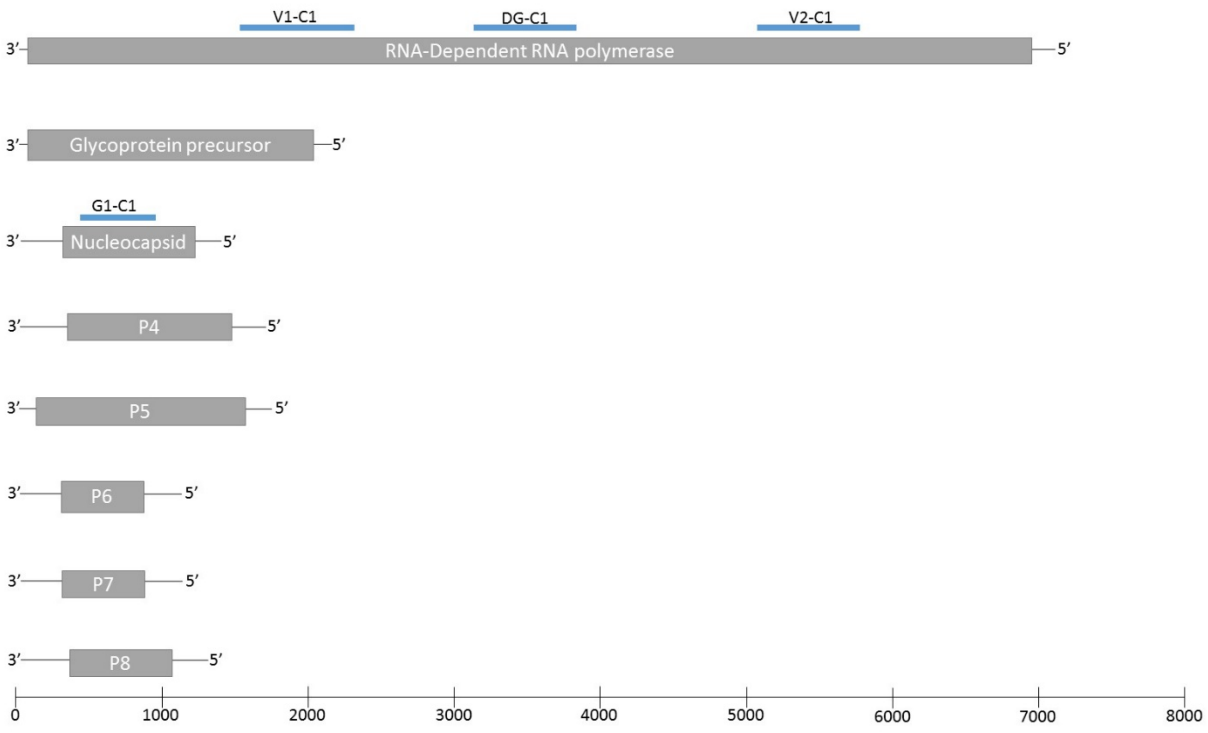

Figure 10. Ti ringspot associated virus sequences (blue lines) obtained by NGS are mapped to the genome of *Raspberry leaf blotch virus*. Grey boxes represent open reading frames (ORFs) and are labeled with their putative function. P4-P8 represent ORFs with unknown function. Scale at bottom is in nucleotides.

further compared and found to produce two different small segments (V1-C1 and V2-C1), with lengths of 390bp and 534bp, and, after BLASTX analysis, both were identified as coming from the RdRp region. Manual analysis of deep sequence reads was done for cross examination purposes. Single reads were subjected to *de novo* assembly, generating a total of 42,564 contigs and an additional 9 contigs (G1-688; 17 reads, G1-1861; 9 reads, G1-2451; 8 reads, G1-7183; 4 reads, G1-8180; 4 reads, G1-11608; 3 reads, G2-920; 32 reads, G2-7064; 4 reads, G2-7574; 4 reads) were found to be related to emaraviruses. These contigs were further analyzed and found to produce a single 834 bp fragment (G1-C1) that was identified as an emaravirus nucleocapsid (NC) gene using BLASTX analysis (Figure 10). Amino acid sequences of RdRp and NC proteins was compared to all known emaraviruses using LALIGN. Amino acid sequence homology of RdRp ranged from 34.8% to 79.9% and NC protein ranged from 21.5% to 82.9% (Table 5).

Phylogenetic Analyses. The TiRaV sequences obtained by NGS as well as known and tentative emaraviruses were analyzed to determine their phylogenetic relationships. Phylogenetic trees constructed with amino acid sequences showed a consistent grouping of TiRaV with RLBV and *High Plains wheat mosaic virus* (HPWMoV) when using maximum-likelihood (ML) and neighbor-joining (NJ) algorithms (Figure 11). Phylogenetic trees constructed from RdRp and NC proteins of TiRaV and other emaraviruses yielded similar topologies by generating two (or three) main clades. The first group includes TiRaV, *Raspberry leaf blotch virus,* and HPWMoV, while the second comprised of the rest, which can be subdivided into two subgroups: A, comprising of *European mountain ash ringspot-associated virus* (EMARaV), and Redbud yellow ringspot-associated virus, and B comprising of Pigeonpea sterility mosaic virus 2 (PPSMV2), *Fig mosaic virus* (FMV), RRV *Rose rosette virus* (RRV), and *Pigeonpea sterility mosaic virus* (PPSMV).

Table 5. Percent amino acid identity of RdRp (black) and nucleocapsid (blue) protein of TiRaV next generation sequences compared to different species from the *Emaravirus* genus: *European mountain ash ringspot-associated virus* (EMARaV; YP_003104764.1, CDL74475.1), *Fig mosaic virus* (FMV; BAM13785.1, BAM13809.1), *Pigeon pea sterility mosaic virus-1* (PPSMV; YP_009237282.1,CUR49052.1), *Pigeon pea sterility mosaic virus-2* (PPSMV-2; CEJ20912.1, YP_009268864.1-), *Raspberry leaf blotch virus* (RLBV; YP_009237274.1, YP_009237266.1), Rose rosette virus (RRV; YP_004327589.1, AMQ49217.1), Redbud yellow ringspot-associated virus (RYRaV; AEO95760.1, AEO88241.1), and *High plains wheat mosaic virus* (HPWMoV; AIK3033.1, YP_009237277.1)

	TiRaV	EMARaV*	FMV*	PPSMV-1*	PPSMV-2	RLBV*	RRV*	RYRaV	HPWMoV*
TiRaV		28.2%	27.2%	26.4%	23.4%	**42.1%**	25.3%	30.5%	37.5%
EMARaV*	45.0%		42.3%	36.6%	41.5%	25.5%	38.6%	46.6%	23.2%
FMV*	38.4%	52.3%		46.7%	82.9%	28.6%	65.8%	41.3%	28.3%
PPSMV-1*	38.0%	53.3%	58.0%		45.6%	25.5%	45.3%	37.9%	23.6%
PPSMV-2	38.8%	50.3$	79.9%	59.0%		28.1%	66.8%	39.4%	23.5%
RLBV*	**53.6%**	40.1%	38.2%	36.2%	39.6%		26.6%	21.5%	36.3%
RRV*	37.8%	53.3%	73.0%	58.6%	73.0%	41.2%		39.0%	22.8%
RYRaV	41.2%	58.1%	51.5%	48.6%	49.9%	43.1%	49.4%		25.0%
HPWMoV*	47.8%	41.4%	36.7%	35.5%	35.0%	48.1%	34.8%	39.2%	

*Current members of the genus *Emaravirus*

Discussion

NGS is a powerful tool that has been effectively used for identification of known and unknown plant viruses (Adams et al., 2009; Hagen et al., 2011; Kreuze et al., 2009). Many NGS platforms such as the Illumina HiSeq approach used in this chapter utilize sequence by synthesis (SBS) technology. SBS technology uses a fluorescently labeled nucleotide which serves as a terminator, so when a single labeled dNTP is added to the nucleic acid chain, the fluorescent dye is imaged to identify the base and is cleaved to allow incorporation of the next nucleotide. This allows to a highly accurate base-by-base sequencing (Illumina, San Diego, CA. 2010). Recent emaraviruses such as RRV, HPWMoV, PPSMV-1, and PPSMV-2, were discovered and sequenced using NGS platforms such as Illumia (Bi et al., 2012; Elbeaino et al., 2014, 2015; Laney et al., 2011). In this chapter, Illlumia (Hi-Seq) was used to increase the amount of sequence information for TiRaV by generating partial sequence of two genomic components, the RdRp (RNA1) and putative nucleocapsid (NC) protein (RNA3). The nucleocapsid protein and chimeric RdRp possessed moderate identities from 38.8% to 53.6% and 23.4% to 42.1% respectively, to members of the genus *Emaravirus*. TiRaV was closest related to RLBV for both the RdRp and NC sequences. Furthermore, phylogenetic analyses revealed that TiRaV formed a separate clade with RLBV and HPWMoV. This may indicate TiRaV along with HPWMoV and RLBV evolved along two distinct lineages. Tatineni et al. (2014) suggest that HPWMoV and RLBV share common ancestors with members of the genera *Orthobunyavirus* and *Tospovirus* for their RdRp and glycoproteins, and *Tospovirus*, *Nairovirus*, and *Orthobunyavirus* for their NC protein. Based on phylogenetic

placement, TiRaV may share these common ancestors as well. Analyses of the additional RdRp and NC sequence data obtained here further support the conclusions drawn in CHAPTER 2 in that TiRaV represents a distinct virus in the

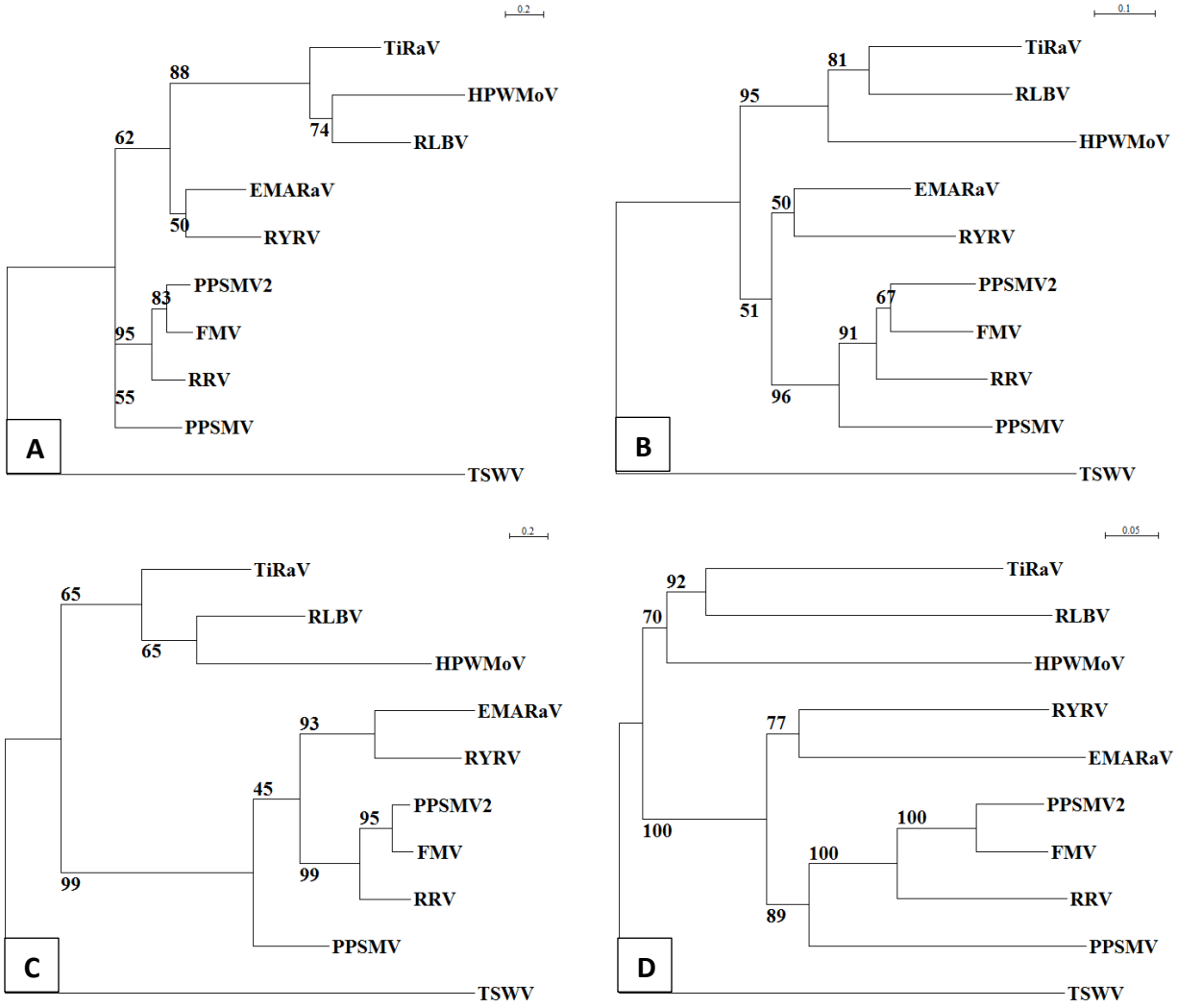

Figure 11. Phylogenetic analysis of TiRaV with maximum-likelihood and neighbor-joining algorithms. Phylograms were constructed with amino acid sequences of RdRp and NC proteins of TiRaV with other members of the genus *Emaravirus*. Maximum-likelihood (A) and neighbor-joining (B) using the partial RdRp sequences; maximum-likelihood (C) and neighbor-joining (D) using NC sequences. Scale on the upper right corner represents the residue substitution per site.

genus *Emaravirus*, and that it is most closely related to RLBV and HPWMoV. TiRaV also appears to follow the same genomic composition of other emaraviruses (Mielke and Muehlbach, 2007; Elbeaino et al., 2009).

Although NGS can generate massive amounts of sequence data, relatively few of the reads obtained in this chapter were of TiRaV origin. Boonham et al. (2014) mentions that methods such as sequencing total mRNA can lead to a massive amount of sequence capacity being wasted due to most of the sequences being of host origin. In this study, however, a dsRNA library was generated for NGS. Such dsRNA libraries are enriched for viral sequences and eliminate much (but not all) of the host genetic material (Al Rwahnih et al. 2015). Two contributing factors may have resulting in the relatively low abundance of TiRaV reads. First, the ti plants from which the library was generated were infected with multiple closteroviruses which appear to replicate to high titers in this host (Melzer et al. 2011, 2013). Indeed, the majority of sequence reads were most closely related to *Cordyline virus 1* (CoV-1), CoV-2, CoV-3, and CoV-4, and these viruses were likely able to outcompete TiRaV during the library construction process. Secondly, TiRaV appears to be a relatively low titer, negative-strand RNA virus that may not produce large quantities of dsRNA. Approaches to increase the number of TiRaV reads in future NGS experiments would be to inoculated ti plants free of other viruses, leaving only the TiRaV dsRNA as the template for the subsequent library construction or increase the number of reads dedicated to the current dsRNA library (Adams et al. 2009). Despite inability to generate a large amount of sequence information on TiRaV, partial genomic sequences corresponding to the RdRp and NC genes of the virus have been generated that may allow for the development of detection assays.

CHAPTER 4

MOLECULAR DETECTION OF TI RINGSPOT ASSOCIATED VIRUS

Ti ringspot associated virus (TiRaV) is potentially a novel emaravirus associated with ti ringspot disease (TRD) of *Cordyline fruticosa*. Like any newly discovered plant pathogen with potentially limited distribution, it is critical to develop specific and robust detection assays to identify infected plants for both management purposes as well as the prevention of long-distance spread of the pathogen via international trade. At present, the only detection method available for TiRaV is the use of emaravirus-specific degenerative primers, followed by sequencing of any amplification product. Difficulties with these primers would occur if there is more than one emaravirus present in a host, which may cause false positive results. It is essential for a specific detection assay to be developed for TiRaV.

Specific primers developed from next generation sequences (NGS) are often utilized in combination with nucleic acid based detection methods, such as reverse-transcription PCR, resulting in highly specific and sensitive detection assays. Members of the genus *Emaravirus* that have had such detection assays developed using this approach include *Fig mosaic virus* (Tzanetakis et al., 2010), *Rose rosette virus* (Laney., 2011), *High Plains wheat mosaic virus* (Tatieni et al. 2014), *Raspberry leaf blotch virus* (McGavin et al., 2012), *Pigeon pea sterility mosaic virus 1* (Elbeaino et al., 2014), and Pigeon pea sterility mosaic virus 2 (Elbeaino et al., 2015).

When the virus titer is low in plant samples, quantitative (q)RT-PCR using TaqMan probes represents an even more sensitive approach; 100-1000 fold more sensitive than conventional PCR, and provides the ability to monitor amplification in real time (Mackay et al.,

2002). Unlike conventional RT-PCR, qRT-PCR requires designing highly specific primers and probes targeting the pathogen of interest.

The specific objectives of this chapter are to utilize NGS data from CHAPTER 3 to develop i) a TiRaV-specific conventional RT-PCR assay and ii) a TiRaV-specifc qRT-PCR assay. Both assays will incorporate a host gene to be used as an internal positive control for the assay(s), in a multiplex format.

Materials and Methods

Sample Collection. Fifty-one samples with symptoms of ti ringspot disease (TRD) were collected from locations across the state including Manoa, Mililani, and Waimanalo from Oahu; Waikapu and Wailuku from Maui; and Imiloa, Hilo, and Kohala from the island of Hawaii. Twenty-three asymptomatic plants were collected from locations across the state including Kekaha from Kauai; Puaulu, Kona, Holualoa, Naalehu, and Hilo from the island of Hawaii; and Lyon Arboretum from Oahu. Samples were labeled and stored at -4°C until processed.

RNA Extraction and cDNA synthesis. RNA extraction and cDNA synthesis was performed as described in *CHAPTER 2* (Figure 12).

Primer Design. Primer Design. TiRaV sequence data obtained by Illumina-based sequencing (Chapter 3) were used to design primers and Taqman® probes for virus detection. Contig (V1C1) from the TiRaV RdRp gene was selected, and primers and probes were designed using PrimerQuest® Tool (IDT, Coralville, USA). To ensure quality control of the detection assay, the Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) gene sequence of *C. fruticosa* (GenBank accession: FJ976127.1) was selected as a housekeeping gene. Primers and probes for its detection were designed using Primer3 (Untergasser et al. 2012). All primers and probes were synthesized by IDT (Table 6).

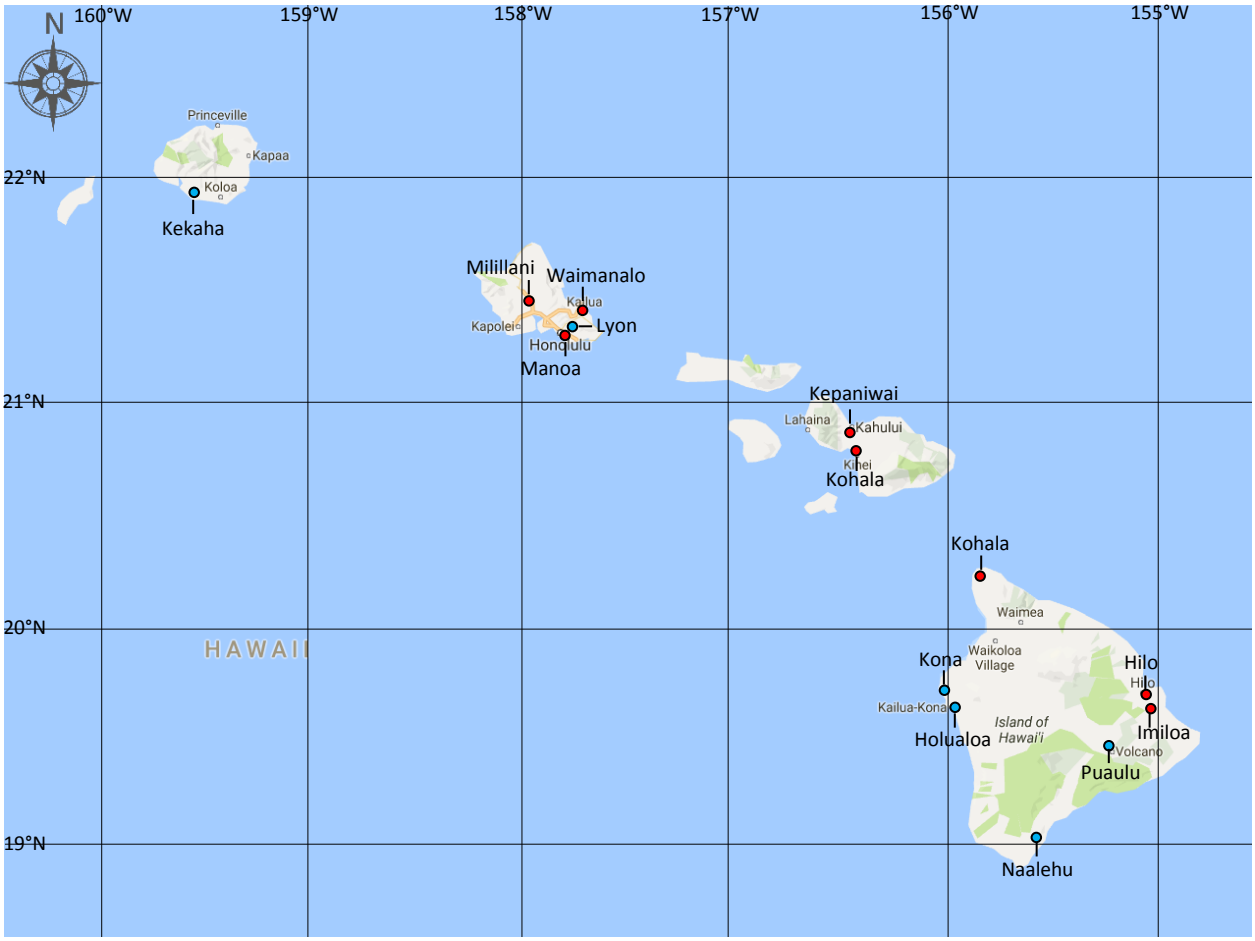

Figure 12. Map of Hawaii showing the locations were symptomatic (Red) and asymptomatic (Blue) tissue samples were collected.

Table 6. TiRaV and RuBisCO primers and probes used in this study.

Name	Sequence (5'-3')	Target
TiRaV FWD	CGC TAA ATA CTG TCA GTT GGT TTC	RdRp
TiRaV REV	AGT TAT AAG GGT GCT AGC CAG T	RdRp
TiRaV Probe	FAM-CAA AGA GTG TGA GTA TCA GTA GTA AGA GGC-3IaBkFQ*	RdRp
RBC L3	**TGG GTT TCT ATG CCA GGT GT**	**RuBisCO**
RBC R3	**AGC AAG ATC ACG TCC CTC AT**	**RuBisCO**
TiRuBisCOp	TET-TAT GCC TGC CCT AAC CGA AA-3BHQ2**	RuBisCO

* IaBkFQ: Iowa Black® FQ Quencher **BHQ2: Black hole quencher 2

Conventional RT-PCR. Preliminary RT-PCR assays were carried out to assess the primer sets. Symptomatic and asymptomatic samples were first tested with RuBisCO primers RBC L3 and RBC R3 for RNA validation. The T_m recommended by the manufacturer was used as the initial annealing temperature. Amplification conditions were: one cycle of 95 °C for 5 mins, thirty-five cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec; and 72 °C for 7 min. PCR products were resolved on a 1% agarose gel and visualized using UV light. After validating the quality of the RNA extraction using the RuBisCO primers, samples were tested for TiRaV using primers TiRaV FWD and TiRaV REV. Amplification involved: one cycle of 95 °C for 5 mins, thirty-five cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec min; and 72 °C for 7 min. PCR products were resolved on a 1% agarose gel and visualized using UV light.

The specific TiRaV and RuBisCO primers were combined to create a single-tube multiplex assay. Initially, 20 µL PCR reactions were created using 10 µL of GoTaq Green Master Mix (Promega), 1 µL of cDNA template, 1 µL of forward (10 pmol) and 1 µL of reverse (10 pmol) TiRaV specific primers, 1 µL of forward (10 pmol) and 1 µL of reverse (10 pmol) RuBisCO primers, and 5 µL of H_2O Synthesized PCR products were resolved on a 1% agarose gel. To optimize the multiplex assay, different primer concentrations and annealing temperatures were evaluated. RuBisCO primers ranging from 5 to 10 pmol were added to the above reaction in 1 pmol increments. The TiRaV specific primer concentrations were not adjusted. Annealing temperatures ranging from 54 °C to 64 °C at 2 °C increments were also evaluated.

Quantitative RT-PCR. Preliminary qRT-PCR assays to assess the primers and probes were conducted in a 25 µL reaction mixture containing 1 µL of cDNA from symptomatic or asymptomatic plants, 12.5 µL GoTAQ Probe Mix (Promega, Madison, Wisconsin), 1 µL (10 pmol) TiRaV FWD, 1 µL (10 pmol) TiRaV REV, 3 µL (30 pmol) probe, and 6.5 µL of H_2O.

qRT-PCR was performed in a Smart Cycler (Cepheid, Sunnyvale, CA) with the following thermal program: one cycle of 95 °C for 2 mins, forty cycles of 95°C for 10 sec, 58°C for 30 sec, 72°C for 30 sec. Optics recorded emitted fluorescence at the 2nd step. Negative controls included a non-template control (NTC) and cDNA from a heathy ti plant. The same protocol was used to separately assess the RuBisCO primers and probe. Fluorescence from FAM and TET reporters and the resultant Ct values were calculated automatically by the Smart Cycler software (Version 1.2d).

To assess the primers and probes in a multiplex assay, four symptomatic samples. A negative control was labeled as NTC. Amplification with both RuBisCO and TiRaV primer/probe sets were carried out in a 25 µL reaction mixture containing 1 µL cDNA of TiRaV (symptomatic/ asymptomatic), 12.5 µL GoTAQ Probe Mix (Promega, Madison, Wisconsin), 1 µL (10 pmol) TiRaV forward primer, 1 µL (10 pmol) TiRaV reverse primer, 1 µL (10 pmol) RuBisCO forward primer, 1 µL (10 pmol) RuBisCO reverse primer, 3 µL (1 pmol) TiRaV probe, 3 µL (1 pmol) RuBisCO probe, and 1.5 µL of H_2O. Optimization of RuBisCO primers were preformed, however no significant changes were seen (data not shown). Amplification was performed in a Smart Cycler with the following thermal program: one cycle of 95 °C for 2 mins, forty cycles of 95°C for 10 sec, 63°C or 30 sec, 72°C for 30 sec. Annealing temperature was set at 63°C per recommendation of the manufacturer. Negative controls (non-template control (NTC) and total RNA from a heathy ti plant). Florescence from FAM reporter and Ct values were calculated automatically from by the Smart Cycler software (Version 1.2d). Another multiplex qRT-PCR reaction with 3 symptomatic and 4 asymptomatic samples was performed using similar amplification conditions.

Results

Conventional RT-PCR. Preliminary evaluation of the TiRaV (TiRaV FwD/ReV) and RuBisCO (RBC L3/RBC R3) primers indicate that all sets successfully amplified their target sequences at the manufacturer's suggested annealing temperature. The TiRaV and RuBisCO primer sets produced the expected amplification products of 213 bp (Figure 13) and 103 bp (Figure 14) respectively. Of the 51 symptomatic samples tested, 43 produced amplification products for RuBisCO. Of these 43 samples that tested positive for RuBisCO, 40 tested positive for TiRaV. All 23 asymptomatic samples showed amplification for RuBisCO, however no asymptomatic samples had any amplification for TiRaV (Table 7).

Initial attempts at the multiplex version of the detection assay resulted in a disproportionate amplification of the targets, with greater amplification of the RuBisCO gene of *C. fruticosa*. To optimize conditions, RuBisCO primer concentrations were reduced at 1pmol intervals. Reducing the amount of RuBisCO primers to 5 pmol each resulted in a more similar band visibility in the multiplex reaction (Figure 15). The TiRaV amplification product appeared faint, so to improve its visibility, annealing temperatures from 54°C - 64°C were evaluated at 2°C intervals (Figure 16). Two products of the correct size were produced in a single reaction with the amplification products of TiRaV and RuBisCO being 103 and 213 bp in size, respectively. Based on this evaluation, the optimum annealing temperature for the multiplex RT-PCR was found to be 60°C (Figure 17). The optimized PCR reaction consisted of 10 µL of GoTaq Green Master Mix (Promega), 1 µL of cDNA template, 1 µL of forward (10 pmol) and 1 µL of reverse (10 pmol) TiRaV specific primers, 0.5 µL of forward (10 pmol) and 0.5 µL of reverse (10 pmol) RuBisCO primers, and 6 µL of H_2O with optimized amplification protocol involving: one cycle of 95 °C for 5 mins, forty cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C

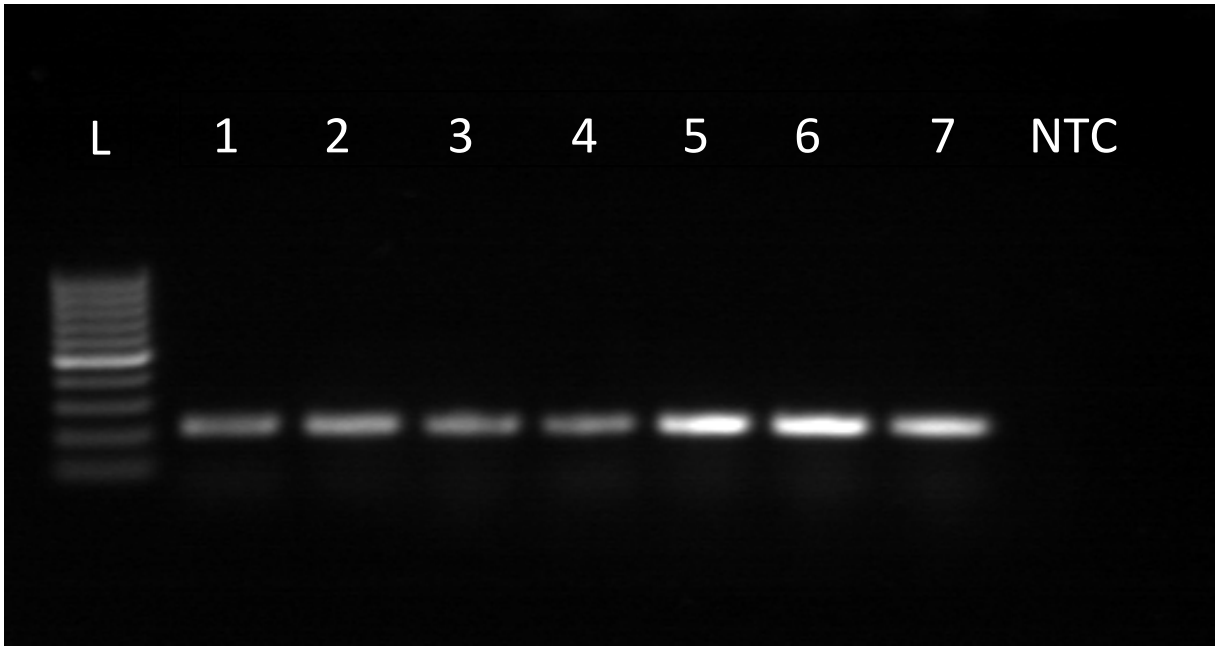

Figure 13. Singleplex RT-PCR of seven symptomatic plants screened with the RuBiSCO primer set. NTC is a non-template (water) control.

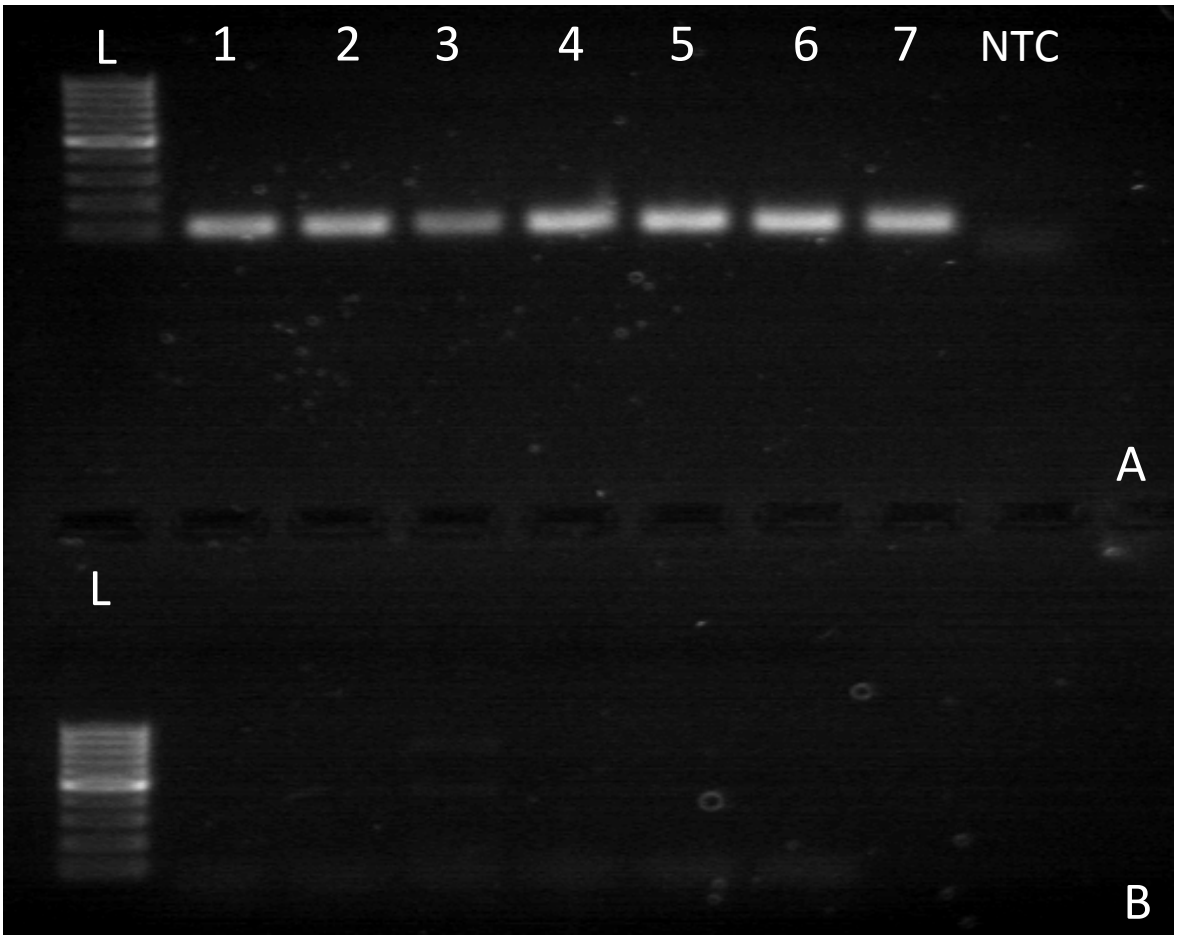

Figure 14. Singleplex RT-PCR of symptomatic (A) and asymptomatic (B) plants screened with the TiRaV primer set. . Symptomatic plants produced the expected 103 bp product while asymptomatic plants did not have any amplification. NTC is a non-template (water) control.

Table 7. Association of TiRaV with ti ringspot disease (TRD) using a TiRaV-specific RT-PCR assay.

TRD Status	Positive for RuBisCO	Positive for TiRaV
Symptomatic	43/51	40/43
Asymptomatic	23/23	0/23

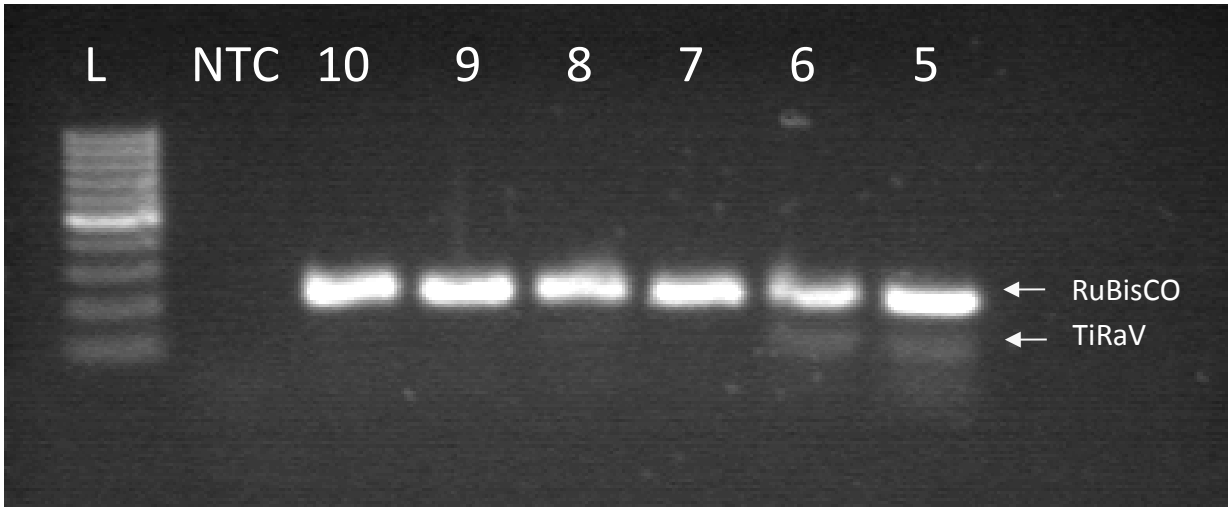

Figure 15. Optimization of conventional multiplex RT-PCR by adjusting RuBisCO primer amounts at 1pmol intervals. NTC is a non-template (water) control.

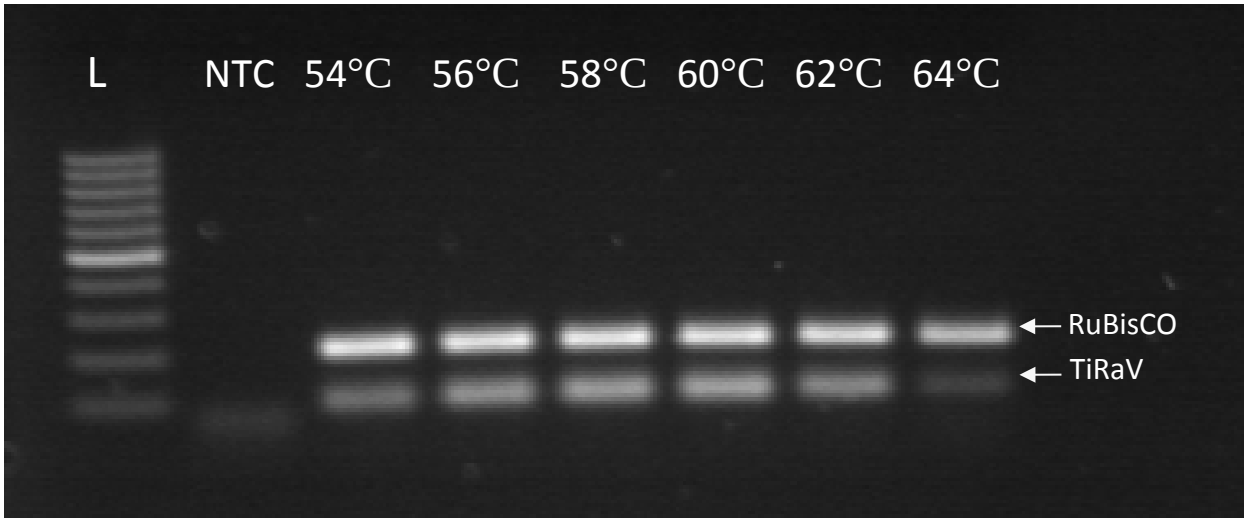

Figure 16. Optimization of conventional multiplex RT-PCR by adjusting the annealing temperature by 2°C intervals. NTC is a non-template (water) control.

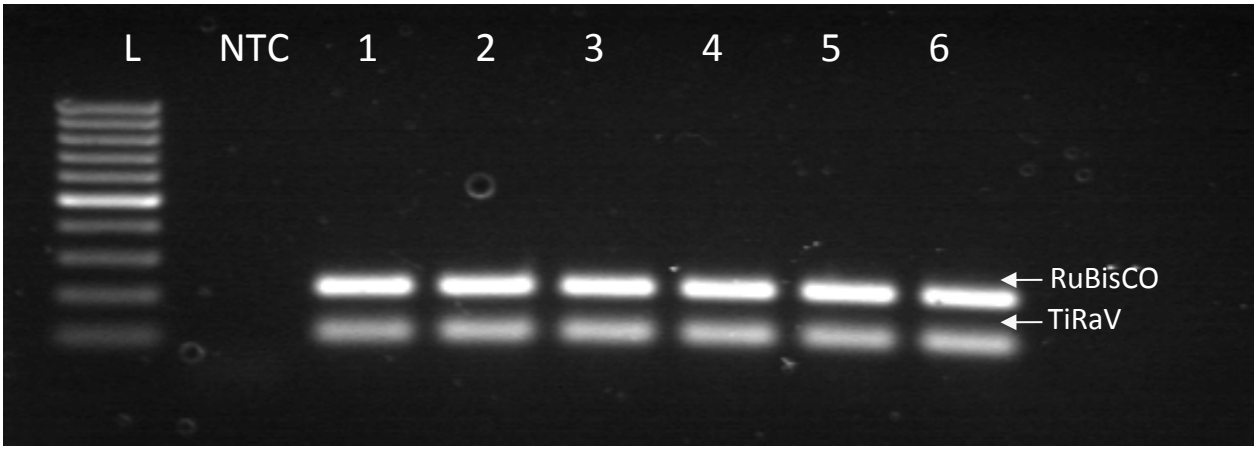

Figure 17. Optimized RT-PCR assay for TiRaV detection using six symptomatic plant samples. NTC is a non-template (water) control.

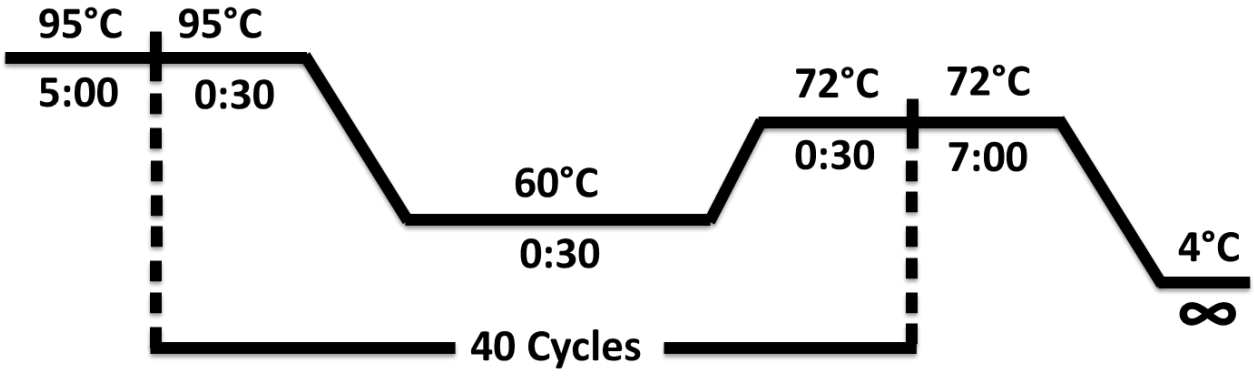

Figure 18. Multiplex RT-PCR conditions

for 30 sec; and 72 °C for 7 min (Figure 18).

Quantitative RT-PCR. Preliminary evaluation of TiRaV and RuBisCO primer/probe in singleplex qRT-PCR reactions suggested that 60°C was a suitable annealing temperature for both primer sets (Figure 19a and 19b). The Ct value of the TiRaV and RuBisCO primer/probe sets were 27.26 and 28.55, respectively. The NTC for both experiments had a Ct value of 0. When multiplexed, the two primer/probe sets were amplified at an optimal temperature of 63°C (Figure 19C and 19D) (optimal temperatures were discussed with IDT technical support). Four samples infected with TiRaV were selected to evaluate the multiplex qRT-PCR assay. All four were positive for both RuBisCO and TiRaV (Table 8; Figure 20A/B), while the NTC was negative. However, in a subsequent experiment that included three samples that were TiRaV positive and four samples that were thought to be TiRaV negative, all samples produced Ct values of less than 40 for both TiRaV and RuBisCO (Table 9). The non-template controls of this experiment had a Ct value of 0.

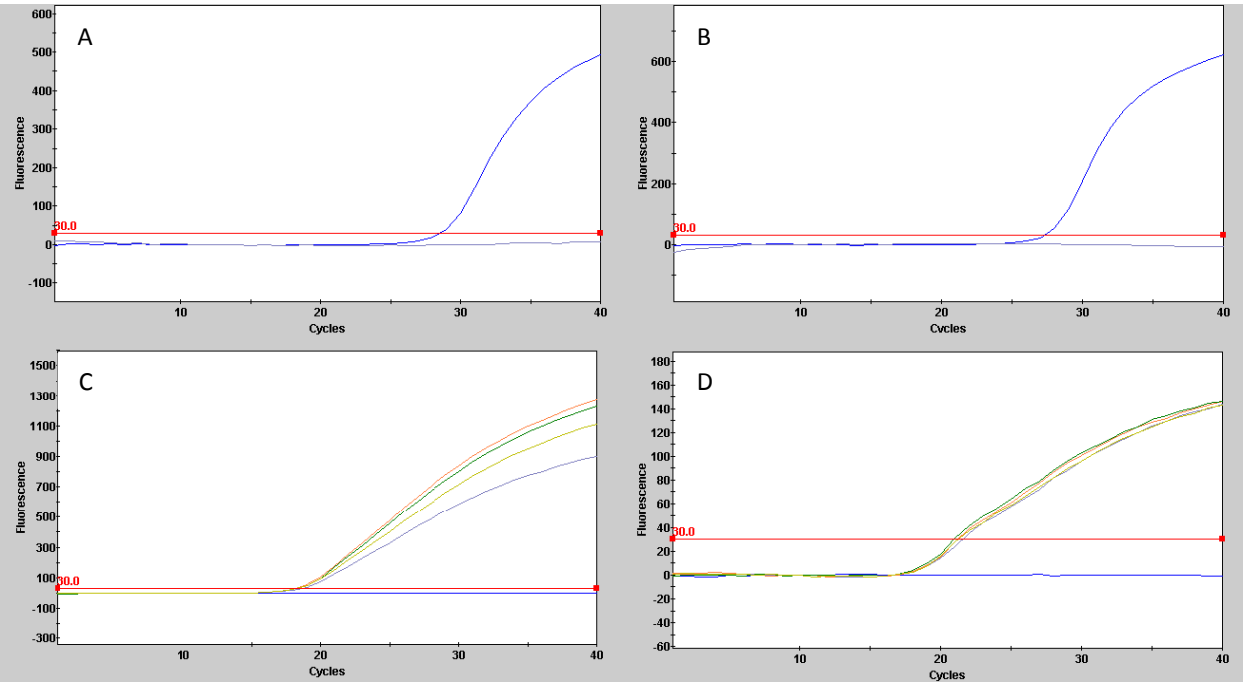

Figure 19. Singleplex qRT-PCR (A & B) and multiplex qRT-PCR (C & D) for TiRaV detection in symptomatic tissues. (A & C) FAM dye channel for the TiRaV primer and probe set. (B & D) TET dye channel for the RuBisCO primer and probe set. Negative controls (Non-Template control) are below the fluorescence threshold.

Table 8. Ct values of symptomatic (Symp) ti samples using the multiplex qRT-PCR targeting TiRaV. NTC is a non-template (water) control.

Sample	RuBisCO (TET)	TiRaV (FAM)
Symp1	21.58	18.52
Symp2	21.18	18.16
Symp3	20.93	18.36
Symp4	21.22	18.33
NTC	0	0

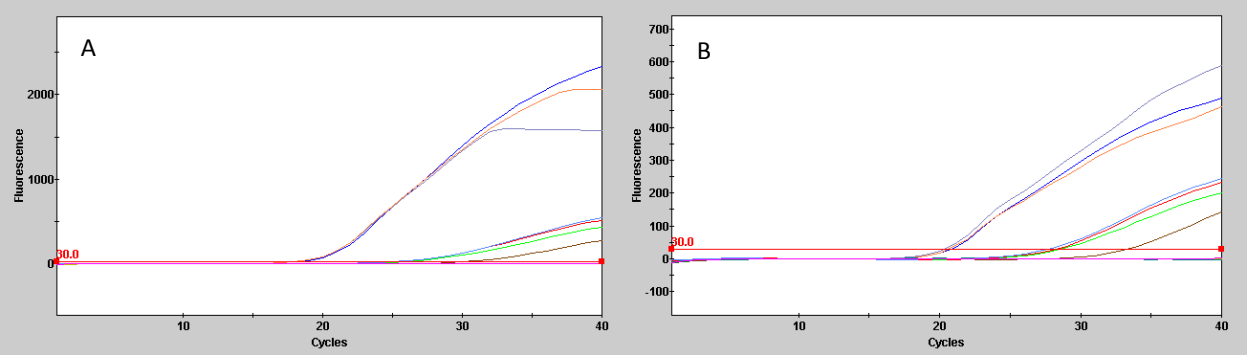

Figure 20. Multiplex qRT-PCR of symptomatic and asymptomatic samples. (A) FAM dye channel for the TiRaV primer and probe set. (B & D) TET dye channel for the RuBisCO primer and probe set. Negative controls (Non-Template control) are below the fluorescence threshold.

Table 9. Ct values of symptomatic (Symp) and asymptomatic (Asymp) ti samples using multiplex qRT-PCR assay targeting TiRaV. NTC is a non-template (water) control.

Sample	RuBisCO (TET)	TiRaV (FAM)
Symp1	20.31	18.47
Symp2	20.36	18.40
Symp3	20.63	18.36
NTC1	0	0
NTC2	0	0
Asymp1	33.31	30.91
Asymp2	28.25	25.74
Asymp3	28.63	26.33
Asymp4	27.74	25.77
NTC1	0	0
NTC2	0	0

Discussion

TiRaV is a newly discovered virus infecting ti plants in Hawaii. The development of TiRaV-specific detection assays is critical to further understand its role in the etiology of TRD, determining the geographical distribution of TiRaV, and the development of management strategies for this virus and TRD. This chapter describes the development and evaluation of two PCR-based TiRaV-specific molecular detection assays: conventional RT-PCR and qRT-PCR. Incorporated into these TiRaV detection assays is the ability to detect the *C. fruticosa* RuBisCO gene, which serves as an internal positive control for the detection assay. The presence of an internal positive control helps to resolve errors that may occur during the testing process, such as total RNA not being successfully extracted, RNA degradation post-extraction, or errors in cDNA synthesis. Any of these situations can result in false negatives or type II errors. For this assay, RuBisCO was selected as an internal positive control gene due to its previous success in assays detecting closteroviruses in *C. fruticosa*. (Melzer et al. 2013). For both the RT-PCR and qRT-PCR assays, detection of TiRaV and the *C. fruticosa* RuBisCO were multiplexed in a single reaction for convenience and to ensure the efficient use of costly PCR reagents.

Using the conventional RT-PCR assay, 51 symptomatic and 23 asymptomatic samples were tested to determine if the assay produced consistent results, as well as provide further evidence of an association between TRD and TiRaV (CHAPTER 2). After the initial RuBisCO screening, 43 of the 51 symptomatic samples and all 23 asymptomatic samples tested positive for RuBisCO, indicating there were problems with RNA isolation, RNA degradation, or cDNA synthesis for ten of the symptomatic samples. These ten samples were excluded from further testing. Of the remaining 43 symptomatic samples, 40 were TiRaV positive whereas none of the asymptomatic samples were TiRaV positive. The three symptomatic samples that were not

amplified may be due to low virus titer or these plants were infected with an isolate of TiRaV or another virus that could not be detected by this assay. Pallas and Garcia (2011) found that virus titer does not necessarily correlate to the severity of the symptoms; indicating that the disease can be the result of specific interactions between virus and host components. A ti plant infected with TiRaV may have low virus titer but can produce symptoms. Thus it is not unexpected that some symptomatic samples would have no visible amplification by conventional RT-PCR.

A qRT-PCR assay was created in an attempt to provide a rapid, highly-sensitive detection method for TiRaV. Preliminary results of the primer/probe sets in singleplex and multiplex assays were encouraging when using symptomatic samples, as Ct values for TiRaV and RuBisCO ranged from 20-30 cycles. However, when in the multiplex format, the four symptomatic samples had considerably lower Ct values than for the singleplex format (Figure 19, Table 8). The reason for this observation is unclear, but may involve the reagent dynamics in the reaction. When asymptomatic samples were tested, Ct values were generated as expected for RuBisCO, but also for TiRaV, indicating these samples were TiRaV positive (Figure 20). The asymptomatic samples had been obtained from ti plants grown from tissue culture and transferred to a greenhouse setting. These plant were previously screened for TiRaV through conventional RT-PCR using both degenerative and specific primers, with both assays indicating the asymptomatic plants were TiRaV-free. All NTC reactions were negative, indicating that contamination was not a factor. The unusual amplification curve of the asymptomatic samples (Figure 20) for both the RuBisCO and TiRaV primer sets as well as the much higher Ct values for RuBisCO in comparison with symptomatic samples suggests further optimization of the qRT-PCR assay is required. Although it was desirable to have a quantifiable multiplex RT- PCR, the conventional multiplex RT-PCR developed provides an effective method for detecting TiRaV.

CHAPTER 5

CONCLUSION AND FUTURE STUDIES

Ti ringspot disease (TRD) is an emerging disease affecting *Cordyline fruticosa* in Hawaii. Double membrane bodies (DMBs) characteristic of the unassigned virus genus *Emaravirus* were previously associated with TRD (Park et al., unpublished), suggesting a possible etiological agent. In this study, degenerative emara-specific primers, sequence homology, and presence of RdRp and nucleocapsid genes provided further evidence for the presence of an emaravirus in ti plants with TRD.

Six viruses are currently members of the genus *Emaravirus.* These viruses are well characterized at the molecular level and show conserved regions in their genome. Degenerative primers targeting highly conversed regions in the RNA-dependent RNA polymerase (RdRp) gene of known emaraviruses (Elbeaino et al 2013) detected a virus in symptomatic tissue and not in asymptomatic tissues, suggesting the virus was the causal agent. Population studies determined there was little genetic divergence within the virus population, indicating only a single virus was being amplified by the degenerative primers. Based on sequence homology, the virus present in ti was found to be most closely related to *Raspberry leaf blotch virus* (RLBV) and *High Plains wheat mosaic virus* (HPWMoV). A difference in homology of more than 25% was seen when compared to all tentative and putative members of the *Emaravirus* genus. Phylogenetic analyses generated a distinct clade that included the ti virus, RLBV, and HPWMoV. Based on these results, it was hypothesized that a new emara-like virus was the causal agent of TRD, and a tentative name for the virus was proposed: ti ringspot associated virus (TiRaV).

The genome of all six current emaravirus members have been well characterized and have similar organization (Mielke-Ehret and Mülbach., 2012; Laney et al., 2011; Serrano et al., 2004; Tatineni et al., 2014; Mcgavin et al., 2012; Kumar et al., 2005). To further understand the TiRaV genome, double-stranded (ds)RNAs representing the replicative form of TiRaV were isolated from ti plants. Next generation sequencing (NGS) of a dsRNA library revealed partial sequences of RdRp and nucleocapsid (NC) genes. Sequence homology and phylogenetic analysis of these sequences provided further evidence supporting TiRaV as a new virus species of virus and member of the *Emaravirus* genus. However, the full-length RdRp and NC genes must be sequenced to confirm the taxonomic placement of TiRaV. Obtaining the full length genomic sequence identify the number of RNA segments the genome of TiRaV is composed of, and compare this number with other established emaraviruses. At present, the reported number of genomic RNA segments varies for different emaravirus (Mielke-Ehret and Mülbach, 2012).

An efficient and reliable reverse-transcription (RT)-PCR assay for the detection of TiRaV was developed that will allow future studies on this virus to proceed. NGS data were utilized to develop specific primers and probes targeting the RdRp region of TiRaV. Symptomatic and asymptomatic ti samples obtained from across the state and assayed using this RT-PCR procedure strengthened the claim that TiRaV is the causal agent of TRD. This assay was multiplexed by including a RuBisCO target used an internal positive control for the assay. A quantitative (q)RT-PCR assay was developed, but appears to need further optimization before it can be considered a reliable assay for TiRaV detection. Future detection work also includes the need for a high-throughput assay such as enzyme-linked immunosorbent assay (ELISA). The development of polyclonal and monoclonal antibodies has been regularly used within the genus with satisfactory results (Jordan et al., 2016; Mielke-Ehret and Mülbach, 2012; Divya et al. 2005;

Forster et al., 2001). Such assays are essential for early detection, diagnosis, and control of new and emerging viruses like TiRaV.

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